

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

(12) **UK Patent Application** (19) **GB** (11) **2 317 891** (13) **A**

(43) Date of A Publication 08.04.1998

(21) Application No 9720890.4

(22) Date of Filing 01.10.1997

(30) Priority Data

| | | |
|----------------|-----------------|---------|
| (31) 08/724643 | (32) 01.10.1996 | (33) US |
| 08/844419 | 18.04.1997 | |
| 08/846017 | 25.04.1997 | |
| 08/851843 | 06.05.1997 | |
| 08/854050 | 09.05.1997 | |
| 08/911312 | 14.08.1997 | |
| 08/912951 | 14.08.1997 | |
| 08/915503 | 14.08.1997 | |

(71) Applicant(s)

Geron Corporation

(Incorporated in USA - California)

230 Constitution Drive, Menlo Park, California 94025,
United States of America

(71), (72) and (74) continued overleaf

(51) INT CL⁶

C12N 9/12, A61K 38/46, C07K 16/18 16/40, C12N
15/54, C12Q 1/34 1/68

(52) UK CL (Edition P)

C3H HB7E H645 H650 H651 H652 H653 H654 H655
H656 H657 H728
C6Y YB YF YG08 YG09 YM Y115 Y125 Y130 Y134 Y156
Y162 Y189 Y192 Y313 Y319 Y327 Y330 Y331 Y332
Y333 Y338 Y405 Y406 Y407 Y410 Y420 Y501 Y503
U1S S1068 S1284 S1285 S1289 S1303 S1313 S1332
S1333 S1334 S1337 S2411 S2415 S2416 S2417 S2419

(56) Documents Cited

WO 96/01835 A1
Science 1997,278,561-567 Science 1997,277,955-959
Trends in Cell Biology 1997,7,299-302 Cell
1997,90,785-795 Current Opinion in Cell Biology
1998,8,374-380 J.Biological Chemistry
1995,270(42),24623-24626 Science 1995,269,1236-1241

(58) Field of Search

UK CL (Edition O) C3H HB7E HB7M
INT CL⁶ C12N 9/12
ONLINE: WPL,CLAIMS,DIALOG/BIOTECH

(54) hTERT, the reverse transcriptase subunit of human telomerase

(57) The polypeptide hTERT (or variants or fragments thereof having at least one biological property of hTERT), antibodies (or fragments thereof) which specifically bind thereto, and nucleic acid sequences encoding hTERT, are described. The polypeptides and nucleic acid sequences are of use in the diagnosis, prognosis and treatment of human disease states (especially cancer and the effect of ageing), in the alteration of the proliferative capacity of cells, and in the identification and screening of compounds for the treatment of such states.

194
193
387

The provisions of paragraph 3 of Schedule 2 to the Patents Rules 1995 have effect in respect of this application, restricting availability of samples of specified micro-organisms to experts in accordance with those provisions.
At least one drawing originally filed was informal and the print reproduced here is taken from a later filed formal copy.
This print takes account of replacement documents submitted after the date of filing to enable the application to comply with the formal requirements of the Patents Rules 1995

GB 2 317 891 A

(71) cont

University Technology Corporation

(Incorporated in USA - Colorado)

**Suite 250, 3101 Iris Avenue, Boulder, Colorado 80301,
United States of America**

(72) Inventor(s)

Thomas R Cech

Joachim Lingner

Toru Nakamura

Karen B Chapman

Gregg B Morin

Calvin B Harley

William H Andrews

(74) Agent and/or Address for Service

Hepworth Lawrence Bryer & Bizley

**Merlin House, Falconry Court, Bakers Lane, EPPING,
Essex, CM16 5DQ, United Kingdom**

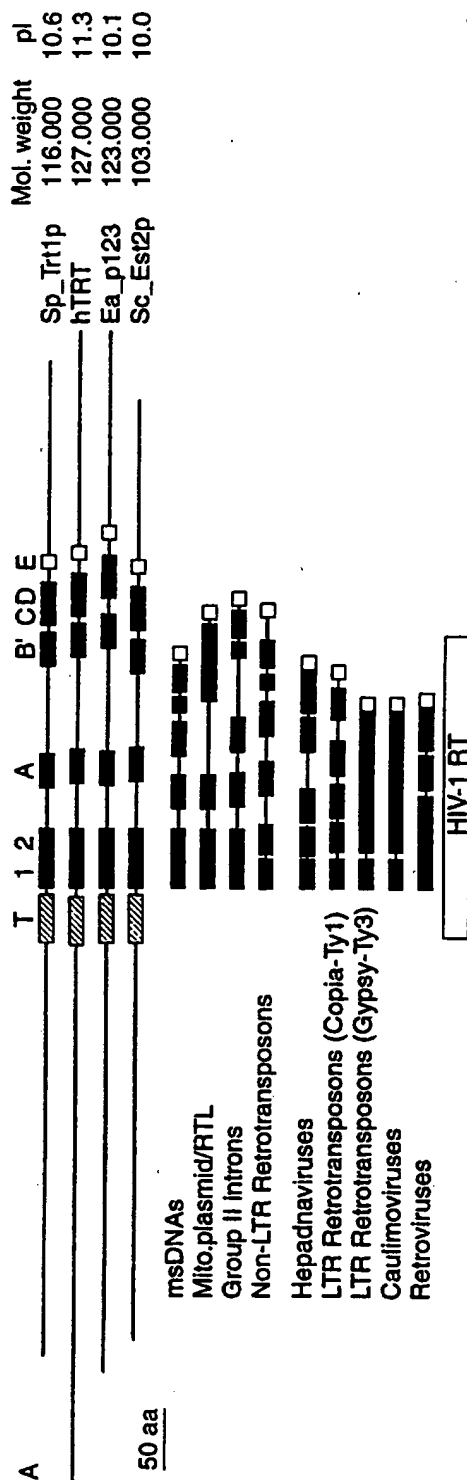


FIG. 2

3/103

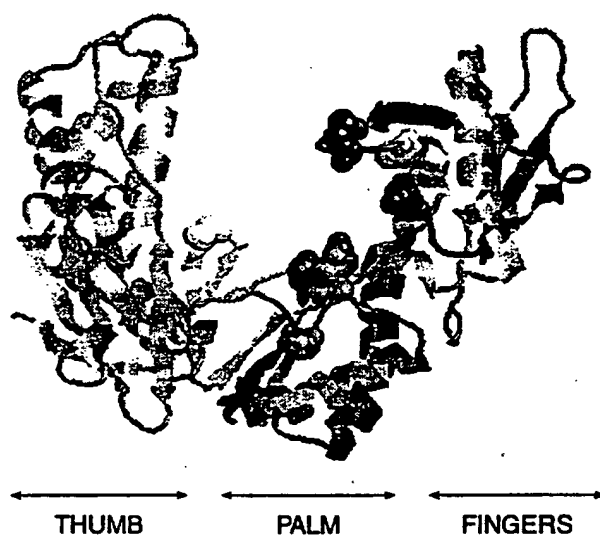


FIG. 3

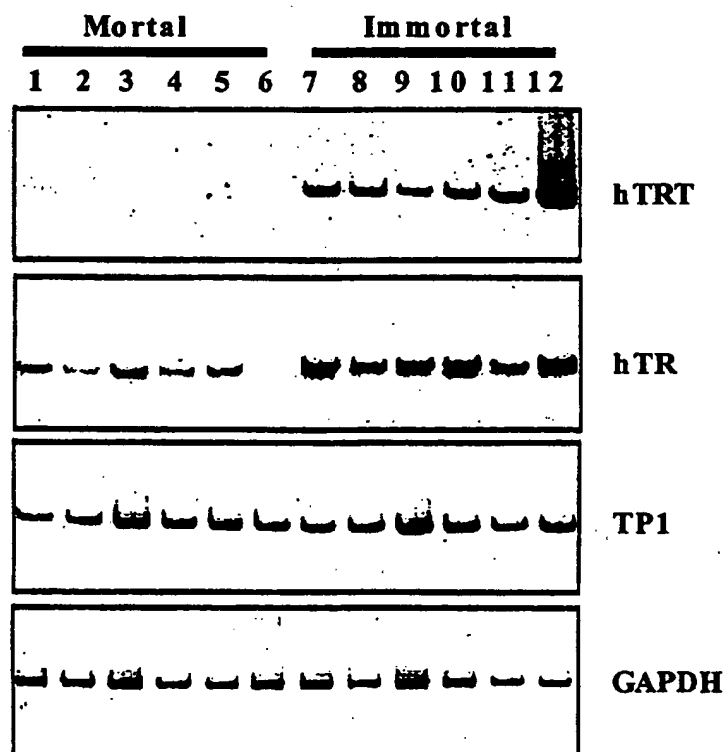


FIG. 5

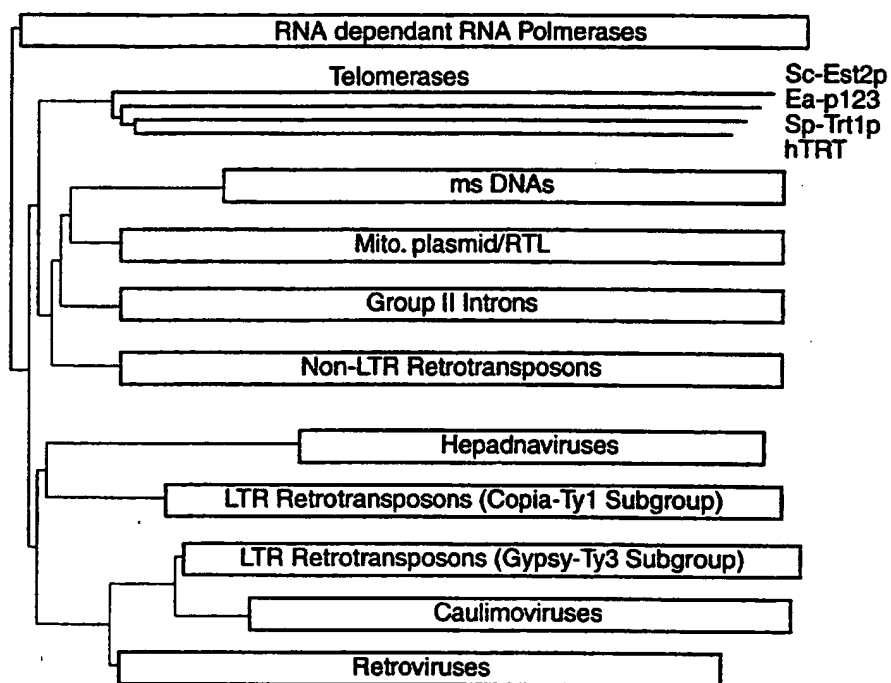


FIG. 6

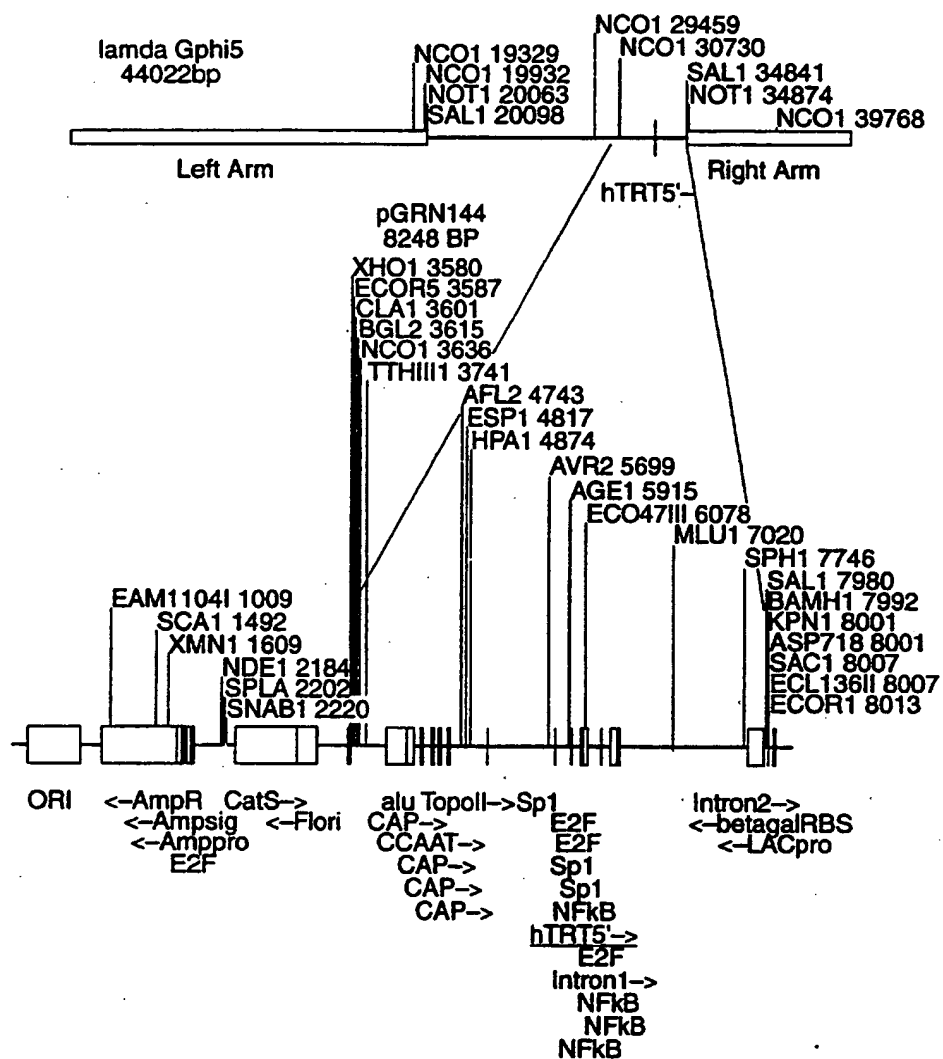


FIG. 7

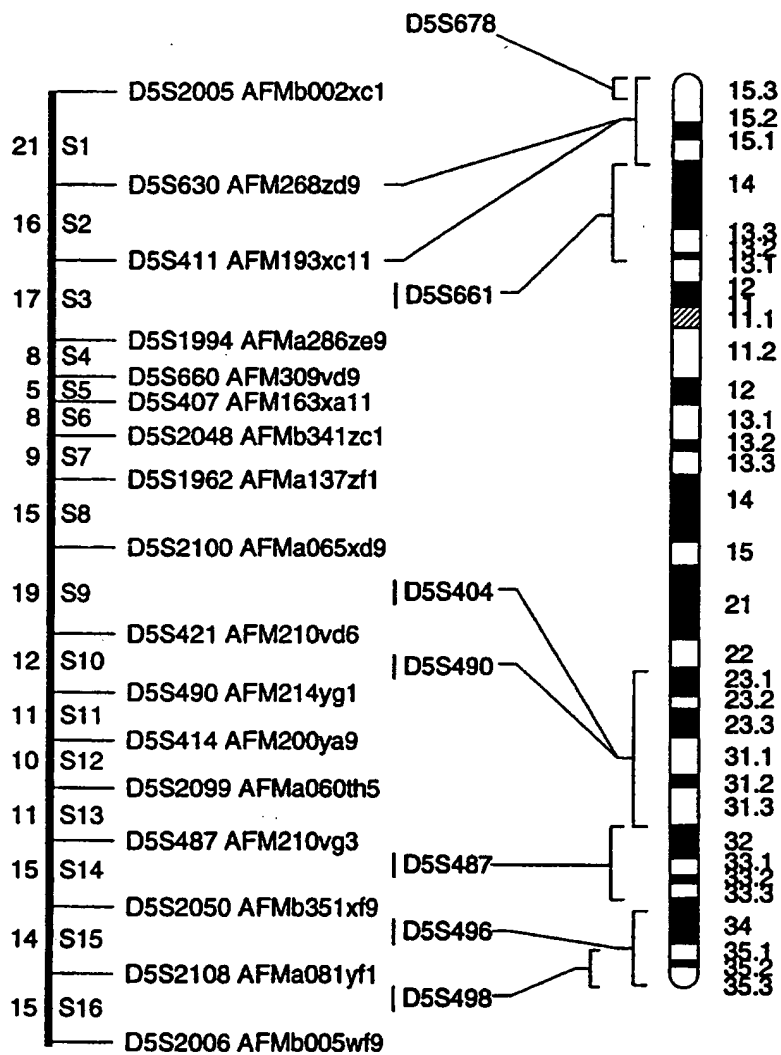


FIG. 8

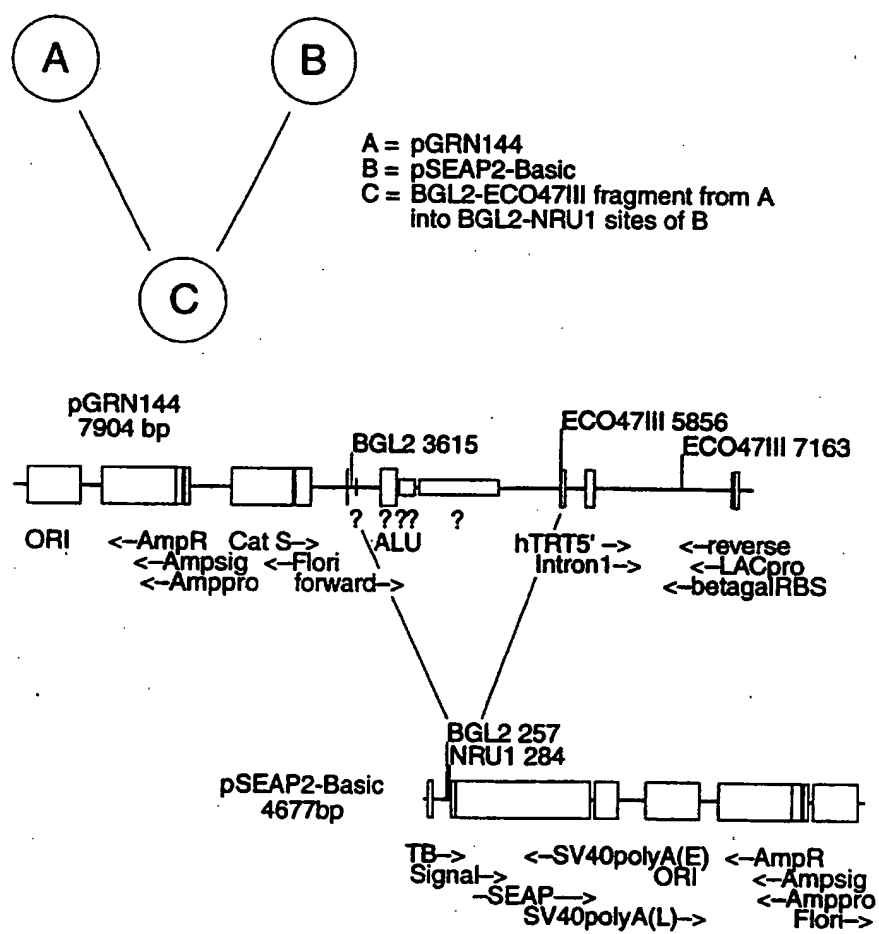


FIG. 9

9/103

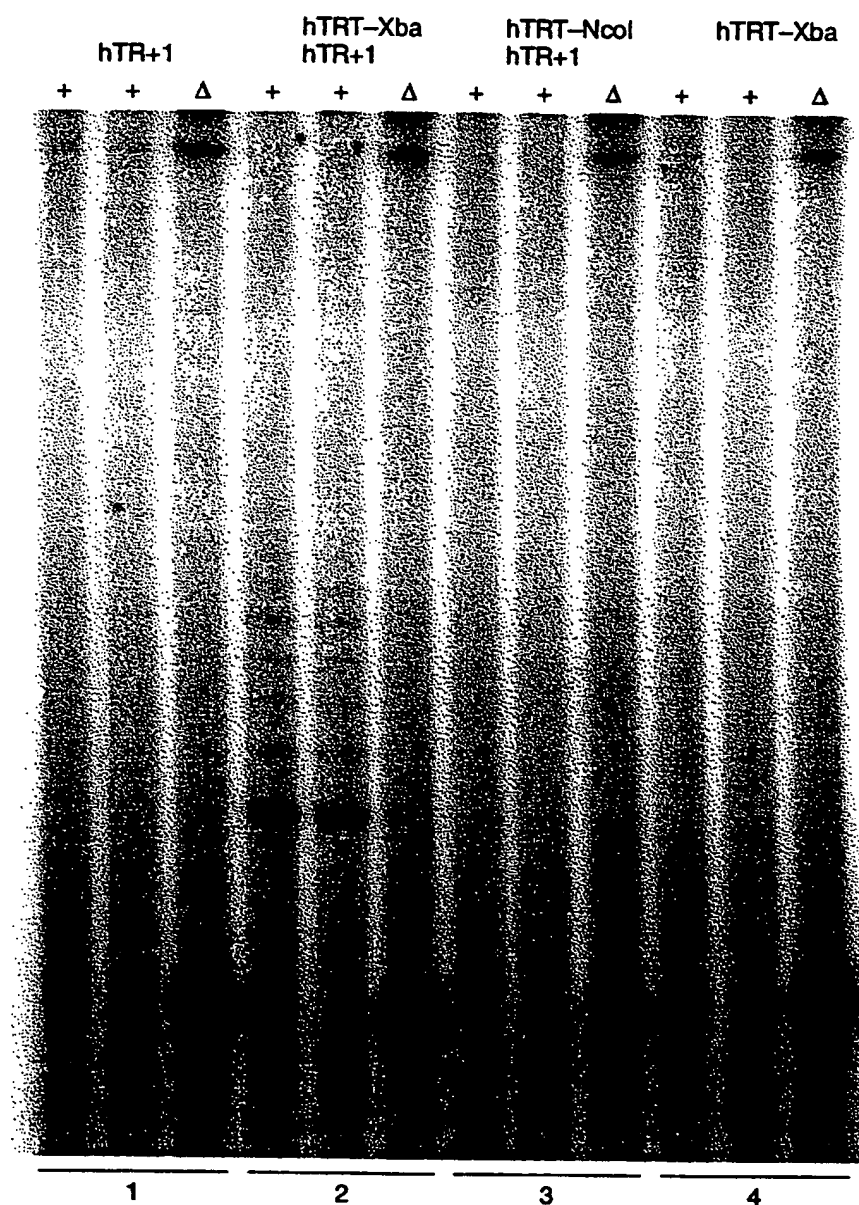


FIG. 10A

10/103

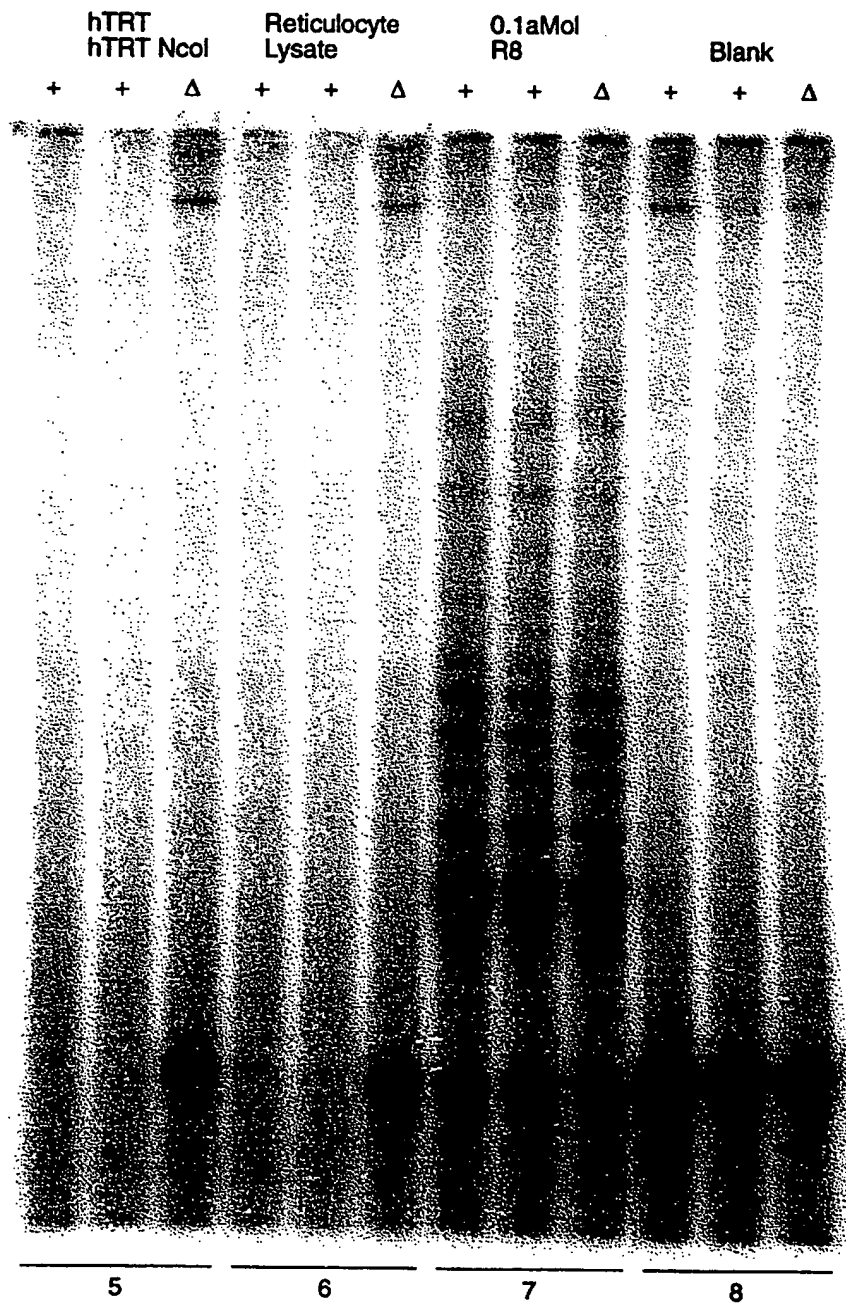


FIG. 10B

Telomerase Specific Motifs

| | | MOTIF T | | MOTIF T' |
|---------|-----|---|------------|----------|
| TRT con | W1 | FFY TE | Y Rk W 1 I | E V |
| hTRT | 546 | WLMVVVVELLRSSFFVTETTFQKRLFFYRKSVMKLSQSIGI | 13 | EAETR |
| spTRT | 429 | WLYNSFIPILOQSFYITESSDLNRRTVFRKDIWKLCPPI | 12 | ENNV |
| Ea_p123 | 441 | WIFEDLVSLIRCFYVTEQQKSYKTYVYRKNINDVIMKMSI | 12 | EKEVE |
| Sc_Est2 | 366 | WLFRLIPKIIQTFFYCTEISSTVT.IVYFRHDTWNLITPFI | 9 | ENNV |

Telomerase RT Motifs (Fingers)

| | MOTIF 1 | MOTIF 2 | MOTIF A | MOTIF B |
|---------|------------------------|----------------------|----------------------------|-------------------|
| TRT con | R ipkk | fr I | p lyf D cyd i | Y q GIPQGS 1S 1 Y |
| hTRT | 11 SRLRFIPKPDG 0 LRPIV | 69 PELYFVKVDVTGAYDTI | 104 YVQCQGIPOGSILSTLLCSLCY | |
| spTRT | 10 AVIRLLPKKNT 0 FRLIT | 66 RKKYFVRIDIKSCYDRI | 99 YLQKVGIPQGSILSSFLCHFYM | |
| Ea_p123 | 10 GKRLIPKKT 0 FRPIM | 67 PKLFFATMDIEKCYDSV | 117 YKQTKGIPQGLCVSSILSSFTY | |
| Sc_Est2 | 13 SKMRIIPKSN 2 FRIIA | 68 PELYFMKFDVKSCYDSI | 85 YIREDGLFQGSLSAPIVDLVY | |
| RT con | p hh h k | hr h | h hDh AF h | hpQG pp hh h |
| | | | GY | |

Telomerase RT Motifs (Palm, Primer Grip)

| | MOTIF C | MOTIF D | MOTIF E |
|---------|---------------------|--------------------|-----------------|
| TRT con | lllr1 DDfL it | g n K | w g s 1 |
| hTRT | 15 LLLRLVDDFLVLT 15 | GVPEYGCWNLKRTTV 24 | WCGLLLDTRTL 192 |
| spTRT | 16 VLLRWVDDFLFIT 15 | GFEKHNFSTLEKTVI 22 | FFGFSVNMRL 176 |
| Ea_p123 | 24 LLMRLTDDYLLIT 15 | VRENGKFNKKLOT 28 | WIGISIDMKTL 174 |
| Sc_Est2 | 18 LILKLADDFLIIS 15 | GFQYNANRDKILA 25 | WKHSSTMNFFH 141 |
| RT con | h Y Ddhhh | Gh h ck h | hLG h |
| | F | | |

FIG. 11

12/103

181 GGACCCGGCGGCTTTCCGCGCGCTGGTGGCCCAGTGCCTGGTGTGCGTGCCCTGGGACGC
CCTGGGCGCGCGAAAGGCGCGGACCACCGGGTCACGGACCACACGCACGGGACCCTGCG

NFkB_CS1
GGGRQTYQC
NFkB-MHC-I.2
TGGGCTTCCC

241 ACGGCCGCCCCCGCGCCCCCTCCTTCCGCCAGGTGGGCCTCCCCGGGGTCGGCGTCCG
TGCCGGCGGGGGCGGCGGGGGAGGAAGGCGGTCCACCCGGAGGGGCCCCAGCCGCAGGC

Intron1

301 GCTGGGGTTGAGGGCGGCCGGGGGAACCAGCGACATGCGGAGAGCAGCGCAGGCGACTC
CGACCCCAACTCCCGCGGCCCCCTTGGTCGCTGTACGCCTCTCGTCGCGTCCGCTGAG

NFkB_CS1
GGGRQTYQC
NFkB_CS2
RGGGRMTYYCC
Topo_II_cleavage_site
RNYNNCNNGYNGKTNINY
*****>

361 AGGGCGCTTCCCCCGCAGGTGTCCTGCCTGAAGGAGCTGGTGGCCCGAGTGCTGCAGAGG
TCCCGCGAAGGGGGCGTCCACAGGACGGACTTCCTCGACCACCGGGCTCACGACGTCTCC

FIG. 12

```

1  AAAACCCCAA AACCCCAAAA CCCCTTTT TAG AGCCCTGCAG TTGGAATAT
51  AACCTCAGTA TTAATAAGCT CAGATTTTAA ATATTAATTA CAAAACCTAA
101 ATGGAGGTTG ATGTTGATAA TCAAGCTGAT AATCATGGCA TTCACCTCAGC
151 TCTTAAGACT TGTGAAGAAA TTAAAGAAGC TAAAACGTTG TACTCTTGGA
201 TCCAGAAAGT TATTAGATGA AGAAATCAAT CTCAAAGTCA TTATAAAGAT
251 TTAGAAGATA TTAATAATATT TGCGCAGACA AATATTGTTG CTACTCCACG
301 AGACTATAAT GAAGAAGATT TTAAAGTTAT TGCAAGAAAA GAAGTATTTT
351 CAACTGGACT AATGATCGAA CTTATTGACA AATGCTTAGT TGAACTTCTT
401 TCATCAAGCG ATGTTTCAGA TAGACAAAAA CTTCAATGAT TTGGATTTCA
451 ACTTAAGGGA AATCAATTAG CAAAGACCCA TTTATTAACA GCTCTTTCAA
501 CTCAAAAGCA GTATTTCTTT CAAGACGAAT GGAACCAAGT TAGAGCAATG
551 ATTGGAAATG AGCTCTTCCG ACATCTCTAC ACTAAATATT TAATATTCCA
601 GCGAACTTCT GAAGGAACCTC TTGTTCAATT TTGCGGGAAT AACGTTTTTG
651 ATCATTTGAA AGTCAACGAT AAGTTTGACA AAAAGCAAAA AGGTGGAGCA
701 GCAGACATGA ATGAACCTCG ATGTTGATCA ACCTGCAAAAT ACAATGTCAA
751 GAATGAGAAA GATCACTTTC TCAACAACAT CAACGTGCCG AATTGGAATA
801 ATATGAAATC AAGAACCAGA ATATTTTATT GCACTCATTG TAATAGAAAT
851 AACCAATTCT TCAAAAAGCA TGAGTTTGTG AGTAACAAAA ACAATATTTC
901 AGCGATGGAC AGAGCTCAGA CGATATTCAC GAATATATTG AGATTTAATA
951 GAATTAGAAA GAAGCTAAAA GATAAGGTTA TCGAAAAAAT TGCCTACATG
1001 CTTGAGAAAAG TCAAAGATTG TAACCTCAAC TACTATTTAA CAAAATCTTG
1051 TCCTCTTCCA GAAAATTGGC GGAACGGAA ACAAAAAATC GAAAACCTGA
1101 TAAATAAAAC TAGAGAAGAA AAGTCGAAGT ACTATGAAGA GCTGTTTAGC
1151 TACACAACCTG ATAATAAATG CGTCACACAA TTTATTAATG AATTTTCTA
1201 CAATATACTC CCCAAAGACT TTTGACTGG AAGAAACCGT AAGAATTTTC
1251 AAAAGAAAGT TAAGAAATAT GTGGAACATA ACAAGCATGA ACTCATTCAC
1301 AAAAAGTTAT TGCTTGAGAA GATCAATACA AGAGAAATAT CATGGATGCA
1351 GGTGAGACC TCTGCAAAGC ATTTTATTA TTTTGATCAC GAAAACATCT
1401 ACGTCTTATG GAAATTGCTC CGATGGATAT TCGAGGATCT CGTCGTCTCG
1451 CTGATTAGAT GATTTTCTA TGTCACCGAG CAACAGAAAA GTTACTCCAA
1501 AACCTATTAC TACAGAAAGA ATATTGGGA CGTCATTATG AAAATGTCAA
1551 TCGCAGACTT AAAGAAGGAA ACGCTTGCTG AGGTCCAAGA AAAAGAGGTT
1601 GAAGAATGGA AAAAGTCGCT TGGATTTGCA CCTGGAAAAC TCAGACTAAT
1651 ACCGAAGAAA ACTACTTTCC GTCCAATTAT GACTTTCAAT AAGAAGATTG
1701 TAAATTGAGA CCGGAAGACT ACAAATTAAC CTACAAATAC GAAGTTATTG
1751 AACTCTCACT TAATGCTTAA GACATTGAAG AATAGAATGT TTAAAGATCC
1801 TTTTGGATTG GCTGTTTTTA ACTATGATGA TGTAATGAAA AAGTATGAGG
1851 AGTTTGTGTTG CAAATGGAAG CAAGTTGGAC AACCAAAACCT CTCTTTGCA
1901 ACTATGGATA TCGAAAAGTG ATATGATAGT GTAAACAGAG AAAAAGTATC
1951 AACATTCCCTA AAAACTACTA AATTACTTTC TTCAGATTTC TGGATTATGA
2001 CTGCACAAAT TCTAAAGAGA AAGAATAACA TAGTTATCGA TTCGAAAAAC
2051 TTTAGAAAGA AAGAAATGAA AGATTATTTT AGACAGAAAT TCAGAAGAT
2101 TGCATTGAA GGAGGACAAT ATCCAACCTT ATTCAAGTGT CTGAAAATG
2151 AACAAAATGA CTTAAATGCA AAGAAAACAT TAATTGTTGA AGCAAAGCAA
2201 AGAAATTATT TTAAGAAAGA TAACCTACTT CAACAGTCA TTAATATTTG
2251 CCAATATAAT TACATTAAC TTAATGGGAA GTTTTATAAA CAAACAAAAG
2301 GAATTCCTCA AGGTCTTTGA GTTTCATCAA TTTTGTATC ATTTTATTAT
2351 GCAACATTAG AGGAAAGCTC CTTAGGATTC CTTAGAGATG AATCAATGAA

```

FIG. 13

```

2401 CCCTGAAAAT CCAAATGTTA ATCTTCTAAT GAGACTTACA GATGACTATC
2451 TTTTGATTAC AACTCAAGAG AATAATGCAG TATTGTTTAT TGAGAAACTT
2501 ATAAACGTAA GTCGTGAAAA TGGATTTAAA TTCAATATGA AGAAACTACA
2551 GACTAGTTTT CCATTAAGTC CAAGCAAATT TGCAAAATAC GGAATGGATA
2601 GTGTTGAGGA GCAAAATATT GTTCAAGATT ACTGCGATTG GATTGGCATC
2651 TCAATTGATA TGAAAACCTCT TGCTTTAATG CCAAATATTA ACTTGAGAAT
2701 AGAAGGAATT CTGTGTACAC TCAATCTAAA CATGCAAAACA AAGAAAGCAT
2751 CAATGTGGCT CAAGAAGAAA CTAAAGTCGT TTTTAATGAA TAACATTACC
2801 CATTTATTTA GAAAGACGAT TACAACCGAA GACTTTGCGA ATAAAACCTCT
2851 CAACAAGTTA TTTATATCAG GCGGTTACAA ATACATGCAA TGAGCCAAAG
2901 AATACAAGGA CCACCTTTAAG AAGAACTTAG CTATGAGCAG TATGATCGAC
2951 TTAGAGGTAT CTAAAATTAT ATACTCTGTA ACCAGAGCAT TCTTTAAATA
3001 CCTTGTGTGC AATATTAAAG ATACAATTTT TGGAGAGGAG CATTATCCAG
3051 ACTTTTTCCT TAGCACACTG AAGCACTTTA TTGAAATATT CAGCACAAAA
3101 AAGTACATT TCAACAGAGT TTGCATGATC CTCAAGGCAA AAGAAGCAAA
3151 GCTAAAAAGT GACCAATGTC AATCTCTAAT TCAATATGAT GCATAGTCGA
3201 CTATTCTAAC TTATTTTGGG AAGTTAATTT TCAATTTTGG TCTTATATAC
3251 TGGGGTTTTG GGGTTTTGGG GTTTTGGGG

```

FIG. 13
(CONTINUED)

```

1 MEVDVDNQAD NHGIHSALKT CEEIKEAKTL YSWIQKVIRC RNQSQSHYKD
51 LEDIKIFAQT NIVATPRDYN EEDFKVIARK EVFSTGLMIE LIDKCLVELL
101 SSSDVSDRQK LQCFGFQKLG NQLAKTHLLT ALSTQKQYFF QDEWNQVRAM
151 IGNELFRHLY TKYLIFORTS EGTLVQFCGN NVFDHLKVND KFDKQKQGA
201 ADMNEPRCCS TCKYNVKNEK DHFLNNINVP NWNMKSRTTR IFYCTHFNRN
251 NQFFKKHEFV SNKNNISAMD RAQTIFTNIF RFNRIKKLK DKVIEKIAYM
301 LEKVDFNFN YYLTKSCPLP ENWRERKQKI ENLINKTREE KSKYYEELFS
351 YTTDNKCVTQ FINEFFYNIL PKDFLTGRNR KNFQKKVKKY VELNKHELH
401 KNLLLEKINT REISWMQVET SAKHFYFDFH ENIYVLWLL RWIFEDLVVS
451 LIRCFYVTE QQKSYSKTY YRKNIWDVIM KMSIADLKE TLAEVQEKEV
501 EEWKSLGFA PGKLRLPKK TTFRPIMTFN KKIVNSDRKT TKLTNTKLL
551 NSHMLKTLK NRMFKDPFGF AVFNYDDVMK KYEEFVCKWK QVGQPKLFFA
601 TMDIEKCYDS VNREKLSTFL KTTKLLSSDF WIMTAQILKR KNNIVIDSKN
651 FRKKEMKDYP RQKFQKIALE GGQYPTLFSV LENEQNDLNA KKTLIVEAKQ
701 RNYFKKDNLL QPVINICQYN YINFNGKFYK QTKGIPQGLC VSSILSSFY
751 ATLEESSLGF LRDESMNPEN PNVNLLMRLT DDYLLITTQE NNAVLFIEKL
801 INVSRENGFK FNMKKLQTSF PLSPSKFAKY GMDSVEEQNI VQDYCDWIGI
851 SIDMKTALM PNINLRIEGL LCTLNLMQT KKASMWLKKK LKSPFLMNNIT
901 HYFRKTITTE DFANKTLNKL FISGGYKYM CAKEYKDHFK KNLAMSSMID
951 LEVSKIIYSV TRAFFKYLVC NIKDTIFGEE HYPDFFLSTL KHFIETSTK
1001 KYIFNRVCM I LKAKEAKLKS DQCQSLIQYD A

```

FIG. 14

FIG. 15

1470 GAT CTC GTT TCT ACT TTT CCT AAT TAC CTT ATA TCT ATA CTT GAG TCA AAA AAT TGG CAA 1529
 129 D L V S T F P N Y L I S I L E S K N W Q 148
 1530 CTT TTG TTA GAA AT gtaataaccggttaagatgttgccacttgcacaaagactgacaagtatag T ATC GGC 1601
 149 L L L E I I G 155
 1602 AGT GAT GCC ATG CAT TAC TTA TTA TCC AAA GGA AGT ATT TTT GAG GCT CTT CCA AAT GAC 1661
 156 S D A M H Y L L S K G S I F E A L P N D 175
 1662 AAT TAC CTT CAG ATT TCT GGC ATA CCA CTT TTT AAA AAT AAT GTG TTT GAG GAA ACT GTG 1721
 176 N Y L Q I S G I P L F K N N V F E E T V 195
 1722 TCA AAA AAA AGA AAG CGA ACC ATT GAA ACA TCC ATT ACT CAA AAT AAA AGC GCC CGC AAA 1781
 196 S K K R K R T I E T S I T Q N K S A R K 215
 1782 GAA GTT TCC TGG AAT AGC ATT TCA ATT AGT AGG TTT AGC ATT TTT TAC AGG TCA TCC TAT 1841
 216 E V S W N S I S I S R F S I F Y R S S Y 235
 1842 AAG AAG TTT AAG CAA G gtaactaataactgttattcctcctaataatttttag AT CTA TAT TTT AAC 1907
 236 K K F K Q D L Y F N 245
 1908 TTA CAC TCT ATT TGT GAT CGG AAC ACA GTA CAC ATG TGG CTT CAA TGG ATT TTT CCA AGG 1967
 246 L H S I C D R N T V H M W L Q W I F P R 265
 1968 CAA TTT GGA CTT ATA AAC GCA TTT CAA GTG AAG CAA TTG CAC AAA GTG ATT CCA CTG GTA 2027
 266 Q F G L I N A F Q V K Q L H K V I P L V 285
 2028 TCA CAG AGT ACA GTT GTG CCC AAA CGT CTC CTA AAG GTA TAC CCT TTA ATT GAA CAA ACA 2087
 286 S Q S T V V P K R L L K V Y P L I E Q T 305
 2088 GCA AAG CGA CTC CAT CGT ATT TCT CTA TCA AAA GTT TAC AAC CAT TAT TGC CCA TAT ATT 2147
 306 A K R L H R I S L S K V Y N H Y C P Y I 325
 2148 GAC ACC CAC GAT GAT GAA AAA ATC CTT AGT TAT TCC TTA AAG CCG AAC CAG GTG TTT GCG 2207
 326 D T H D E K I L S Y S L K P N Q V F A 345
 2208 TTT CTT CGA TCC ATT CTT GTT CGA GTG TTT CCT AAA TTA ATC TGG GGT AAC CAA AGG ATA 2267
 346 F L R S I L V R V F P K L I W G N Q R I 365

FIG. 15
(CONTINUED)

2268 TTT GAG ATA ATA TTA AAA G gtaattgtataaaattttattaccactaacgattttaccag AC CTC GAA ACT 2336
 366 F E I I L K D L E T 375
 2337 TTC TTG AAA TTA TCG AGA TAC GAG TCT TTT AGT TTA CAT TAT TTA ATG AGT AAC ATA AAG 2396
 376 F L K L S R Y E S F S L H Y L M S N I K 395
 2397 gtaatatgccaaattttttaccatttaataacaatcag ATT TCA GAA ATT GAA TGG CTA GTC CTT GGA 2465
 396 I S E I E W L V L G 405
 2466 AAA AGG TCA AAT GCG AAA ATG TGC TTA AGT GAT TTT GAG AAA CCG AAG CAA ATA TTT GCG 2525
 406 K R S N A K M C L S D F E K R K Q I F A 425
 2526 GAA TTC ATC TAC TGG CTA TAC AAT TCG TTT ATA ATA CCT ATT TTA CAA TCT TTT TTT TAT 2585
 426 E F I Y W L Y N S F I I P I L Q S F F Y 445
 2586 ATC ACT GAA TCA AGT GAT TTA CGA AAT CGA ACT GTT TAT TTT AGA AAA GAT ATT TGG AAA 2645
 446 I T E S S D L R N R T V Y F R K D I W K 465
 2646 CTC TTG TGC CGA CCC TTT ATT ACA TCA ATG AAA ATG GAA GCG TTT GAA AAA ATA AAC GAG 2705
 466 L L C R P F I T S M K M E A F E K I N E 485
 2706 gtaatttaaaagtattttttgcaaaaagctaataattttcag AAC AAT GTT AGG ATG GAT ACT CAG AAA ACT 2775
 486 N N V R M D T Q K T 495
 2776 ACT TTG CCT CCA GCA GTT ATT CGT CTA TTA CCT AAG AAG AAT ACC TTT CGT CTC ATT ACG 2835
 496 T L P P A V I R L L P K K N T F R L I T 515
 2836 AAT TTA AGA AAA AGA TTC TTA ATA AAG gtattaatttttggtcacatgaatgtactttacttctaatctatta 2906
 516 N L R K R F L I K 524
 2907 ttagcag ATG GGT TCA AAC AAA AAA ATG TTA GTC AGT ACG AAC CAA ACT TTA CGA CCT GTG 2967
 525 M G S N K K M L V S T N Q T L R P V 542
 2968 GCA TCG ATA CTG AAA CAT TTA ATC AAT GAA GAA AGT AGT GGT ATT CCA TTT AAC TTG GAG 3027
 543 A S I L K H L I N E E S S G I P F N L E 562
 3028 GTT TAC ATG AAG CTT CTT ACT TTT AAG AAG GAT CTT CTT AAG CAC CGA ATG TTT GG gtaat 3088
 563 V Y M K L L T F K K D L L K H R M F G 581

FIG. 15
(CONTINUED)

3089 tatataatgcgcgattcctcatttatttgcag G CGT AAG AAG TAT TTT GTA CGG ATA GAT ATA 3155
 582 R K K Y F V R I D I 591
 3156 AAA TCC TGT TAT GAT CGA ATA AAG CAA GAT TTG ATG TTT CGG ATT GTT AAA AAG AAA CTC 3215
 592 K S C Y D R I K Q D L M F R I V K K L 611
 3216 AAG GAT CCC GAA TTT GTA ATT CGA AAG TAT GCA ACC ATA CAT GCA ACA AGT GAC CGA GCT 3275
 612 K D P E F V I R K Y A T I H A T S D R A 631
 3276 ACA AAA AAC TTT GTT AGT GAG GCG TTT TCC TAT T gtaagttatttttcatggaatttttaacaa 3343
 632 T K N F V S E A F S Y F 643
 3344 attcttttttag TT GAT ATG GTG CCT TTT GAA AAA GTC GTG CAG TTA CTT TCT ATG AAA ACA 3405
 644 D M V P F E K V V Q L L S M K T 659
 3406 TCA GAT ACT TTG TTT GTT GAT TTT GTG GAT TAT TGG ACC AAA AGT TCT TCT GAA ATT TTT 3465
 660 S D T L F V D F V D Y W T K S S E I F 679
 3466 AAA ATG CTC AAG GAA CAT CTC TCT GGA CAC AAT GTT AAG gtataccaattgtgaattgtaataaca 3532
 680 K M L K E H L S G H I V K 692
 3533 ctaatgaactag ATA GGA AAT TCT CAA TAC CTT CAA AAA GTT GGT ATC CCT CAG GGC TCA 3593
 693 I G N S Q Y L Q K V G I P Q G S 708
 3594 ATT CTG TCA TCT TTT TTG TGT CAT TTC CAT ATG GAA GAT TTG ATT GAT GAA TAC CTA TCG 3653
 709 I L S S F L C H F Y M E D L I D E Y L S 728
 3654 TTT ACG AAA AAG AAA GGA TCA GTG TTG TTA CGA GTA GTC GAC GAT TTC CTC TTT ATA ACA 3713
 729 F T K K G S V L L R V V D D F L F I T 748
 3714 GTT AAT AAA AAG GAT GCA AAA AAA TTT TTG AAT TTA TCT TTA AGA G gtgagttgtgtcattcc 3777
 749 V N K K D A K K F L N L S L R G 764
 3778 taagttctaaccgttgaag GA TTT GAG AAA CAC AAT TTT TCT ACG AGC CTG GAG AAA ACA GTA 3840
 765 F E K H N F S T S L E K T V 778
 3841 ATA AAC TTT GAA AAT AGT AAT GGG ATA ATA AAC AAT ACT TTT TTT TTT AAT GAA ACC AAG AAA 3900
 779 I N F E N S N G I I N N T F F N E S K K 798

FIG. 15
(CONTINUED)

3901 AGA ATG CCA TTC TTC GGT TTC TCT GTG AAC ATG AGG TCT CTT GAT ACA TTG TTA GCA TGT 3960
 799 R M P F F G F S V N M R S L D T L L A C 818
 3961 CCT AAA ATT GAT GAA GCC TTA TTT AAC TCT ACA TCT GTA GAG CTG ACG AAA CAT ATG GGG 4020
 819 P K I D E A L F N S T S V E L T K H M G 838
 4021 AAA TCT TTT TTT TAC AAA ATT CTA AG gtatactgtgtaactgaataataatcag A TCG 4089
 839 K S F F Y K I L R S 848
 4090 AGC CTT GCA TCC TTT GCA CAA GTA TTT ATT GAC ATT ACC CAC AAT TCA AAA TTC AAT TCT 4149
 849 S L A S F A Q V F I D I T H N S K F N S 868
 4150 TGC TGC AAT ATA TAT AGG CTA GGA TAC TCT ATG TCT ATG AGA GCA CAA GCA TAC TTA AAA 4209
 869 C C N I Y R L G Y S M C M R A Q A Y L K 888
 4210 AGG ATG AAG GAT ATA TTT ATT CCC CAA AGA ATG TTC ATA ACG G gtgagtacttattttaactaga 4274
 889 R M K D I F I P Q R M F I T D 903
 4275 aaagtcattaattaaccccttag AT CTT TTG AAT GTT ATT GGA AGA AAA ATT TGG AAA AAG TTG GCC 4339
 904 L L N V I G R K I W K K L A 917
 4340 GAA ATA TTA GGA TAT ACG AGT AGG CGT TTC TTG TCC TCT GCA GAA GTC AAA TG gtacgtgtc 4401
 918 E I L G Y T S R F L S S A E V K W 935
 4402 ggtctcgagacttcagcaatattgacacacacag G CTT TTT TGT CTT GGA ATG AGA GAT GGT TTG AAA 4468
 936 L F C L G M R D G L K 946
 4469 CCC TCT TTC AAA TAT CAT CCA TGC TTC GAA CAG CTA ATA TAC CAA TTT CAG TCA TTG ACT 4528
 947 P S F K Y H P C F E Q L I Y Q F Q S L T 966
 4529 GAT CTT ATC AAG CCG CTA AGA CCA GTT TTG CGA CAG GTG TTA TTT TTA CAT AGA ATA 4588
 967 D L I K P L R P V L R Q V L F L H R R I 986
 4589 GCT GAT TAA tgtcattttcaatttattattatatacatcctttattactgggtgtttaaacaataattattactaagtata 4665
 987 A D *

FIG. 15
(CONTINUED)

FIG. 15
(CONTINUED)

```

1  gcagcgctgc  gtctctgtgc  gcacgtggga  agccctggcc  ccggccaccc  ccgcatgccc
61  ggcgctcccc  cgctgcgag  ccgtgcgctc  cctgctgcgc  agccactacc  gcgaggtgct
121  gccgctggcc  acgttcgtgc  ggccgctggg  gccccagggc  tggcggtggt  tgcagcgagg
181  ggacccggcg  gctttccgcg  cgctgggtgg  ccagtgcctg  gtgtgcgtgc  cctgggacgc
241  acggccgccc  cccgcccggc  cctccttccg  ccaggtgtcc  tgctgaagg  agctgggtgg
301  ccgagtgtct  cagaggctgt  gcgagcgagg  cgcaagaac  gtgtggcct  tgggtctgc
361  gctgtggac  ggggcccggc  ggggcccccc  cgaggccttc  accaccagcg  tggcagcta
421  cctgcccac  acggtgacc  acgactgcg  ggggagcggg  gcgtgggggc  tgctgtcgcg
481  ccgctgggg  gacgacgtgc  tggttcacct  gctggcacgc  tgcgctct  ttgtgtggt
541  ggctcccagc  tgcgctacc  aggtgtgagg  gccgctgct  taccagctgc  gcgctggcac
601  tcaggccgg  ccccgccac  acgtagtgg  acccgagg  cgtctgggat  gcgaaccggc
661  ctggaacat  agcgtcagg  aggcgggggt  cccctgggc  ctgacagcc  cgggtgag
721  gaggcgagg  ggcagtgcca  gccgaagtct  gccgttgccc  aagaggccca  ggcgtggcgc
781  tgccctggg  ccggagcgg  ccgcccgttg  gcagggttcc  tgggcccacc  cgggagcagc
841  gcgtggacc  agtgaccgtg  gttctgtgt  ggtgtcacct  gccagaccgc  ccgaagaagc
901  cacctcttt  gagggtgctc  tctctggcac  gcgcaactcc  caccatccg  tgggcccaca
961  agcccggtg  ggcccccat  ccacatcgcg  gccaccagc  ccctgggaca  cgccttgtcc
1021  cccggtgtac  gccgagacca  agcacttct  ctactcctca  ggcgacaagg  agcagctgcg
1081  gccctccttc  ctactcagct  ctctgaggcc  cagcctgact  ggcgtcggg  ggctcgtgga
1141  gacctcttt  ctgggttcca  ggccctggat  gccagggact  ccccgagg  tgccccct
1201  gccccagcgc  tactggcaaa  tggggccct  gtttctggag  ctgcttggg  accacgcgca
1261  gtgcccctac  ggggtgtctc  tcaagacgca  ctgcccgtg  cgagctggcg  tcacccagc
1321  agcccggtg  tgtgcccgg  agaagcccc  gggctctgtg  cgggcccccc  aggagaggga
1381  cacagacccc  cgtcgctgtg  tgcagctgct  ccgcccagc  agcagccct  ggcaggtgta
1441  cggctctgt  cgggcctgct  tgcgcccgt  ggtgcccaca  ggctctggg  gctccaggca
1501  caacgaacgc  cgcttctca  ggaacaccaa  gaagtctac  tccctgggga  agcatgccaa
1561  gctctcgtg  caggagctga  cgtggaagat  gagcgtgcg  gactgcgct  ggctgcagc
1621  gagccaggg  gttggctgtg  ttccggcgc  agagcacct  ctgctgagg  agatcctggc
1681  caagtctct  cactggctga  tgagtgtga  cgtcgtcag  ctgctcagg  ctttcttta
1741  tgtcacggag  accacgttt  aaaagaacag  gctcttttc  taccggaaga  gtgtctggag
1801  caagtgtcaa  agcattgga  tcagacagca  ctgaaagg  gtgcagctgc  gggagctgtc
1861  ggaagcagag  gtcaggcagc  atcggaagc  caggcccgc  ctgctgact  ccagactccg
1921  cttcatccc  aagcctgacg  ggctgcggcc  gattgtgaac  atggactacg  tgctgggagc
1981  cagaacgttc  cgcagagaaa  agaggccga  gcgtctcacc  tcgagggtga  aggcactgtt
2041  cagcgtgct  aactacgagc  gggcgccgg  ccccgccct  ctggggcct  ctgtgtggg
2101  cctggagcat  atccacagg  ctggcgcac  cttcgtgtg  cgtgtgcgg  cccaggaccc
2161  gccgctgag  ctgtacttt  tcaaggtgga  tgtgacggg  gcgtacgaca  ccatcccca
2221  ggacaggctc  acggaggta  tcgccagcat  catcaaaccc  cagaacacgt  actgcgtgcg
2281  tcggtatgcc  gtggtccaga  agccgcgcca  tgggacagtc  cgcaaggcct  tcaagagcca
2341  cgtctctacc  ttgacagacc  tccagccgt  catgagacag  ttctgtggc  acctgcagga
2401  gaccagcccc  ctgagggatg  ccgtcgtcat  cgagcagagc  tcctccctga  atgaggccag
2461  cagtggcctc  ttcgacgtct  cctacgctt  catgtgccac  cagccgtgc  cactcagggc
2521  caagtcttac  gtccagtgcc  aggggatccc  gcagggtcc  atcctctcca  cgctgctctg
2581  cagcctgtgc  tacggcgaca  tggagaacaa  gctgtttgc  gggattcggc  gggaggggtg
2641  gctcctgcgt  ttggtggatg  atttctgtt  ggtgacacct  caccctaccc  acgcgaaaac
2701  cttcctcagg  accctggtcc  gaggtgtccc  tgagtatgg  tgcgtggtga  acttgcggaa
2761  gacagtgggt  aacttccctg  tagaagacga  ggccttgggt  ggcacggctt  ttgttcagat
2821  gccggccccc  ggcctattcc  cctggtgcgg  cctgctgctg  gatacccgga  ccttgagggt
2881  gcagagcgac  tactccagct  atgcccggac  ctccatcaga  gccagtctca  ccttcaaccg
2941  cggcttcaag  gctgggagga  acatgcgtcg  caaactctt  ggggtcttgc  ggctgaagt
3001  tcacagcctg  tttctggatt  tgcagggtga  cagcctccag  acggtgtgca  ccaacatcta
3061  caagatcctc  ctgctgcagg  cgtacaggtt  tcacgcatgt  gtgctgcagc  tccatttca
3121  tcagcaagtt  tggaaagacc  ccacatttt  cctgcgcgtc  atctctgaca  cggcctccct
3181  ctgctactcc  atcctgaaag  ccaagaacgc  agggatgtcg  ctgggggcca  agggcgccgc
3241  cggccctctg  ccctccagg  ccgtgcagtg  gctgtgccac  caagcattcc  tgctcaagct
3301  gactcgacac  cgtgtcacct  acgtgccact  cctgggggtca  ctgaggacag  cccagacgca
3361  cctgagtcgg  aagctccgg  ggacgacgct  gactgccctg  gaggccgag  ccaaccggc
3421  actgccctca  gacttcaaga  ccatcctgga  ctgatggcca  cccgcccaca  gccaggccga
3481  gagcagacac  cagcagccct  gtcagcccg  gctctacgtc  ccaggaggag  agggcgccg
3541  cacacccagg  cccgcaccgc  tgggagctg  aggcctgagt  gagtgtttg  ccgagggcgt
3601  catgtccgg  tgaaggctga  ggtccggct  gaggcctgag  cgagtgtcca  gccagggtg
3661  gagtgtccag  cacacctgcc  gtcttcaact  ccccacaggc  tggcgctcgg  ctccaccca
3721  gggccagctt  ttcttcacca  ggagcccggc  ttccactccc  cacataggaa  tagtccatcc
3781  ccagattcgc  cattgttcac  cctcgccct  gccctccttt  gcttccacc  cccacatcc
3841  aggtggagac  cctgagaagg  acctggggag  ctctgggaat  ttggagtgc  caaaggtgtg
3901  ccctgtacac  aggcgaggag  cctgcacctg  gatgggggtc  cctgtgggtc  aaattggggg
3961  gaggtgtgtg  gggagtaaaa  tactgaatat  atgagttttt  cagttttgaa  aaaaa

```

FIG. 16

22/103

MPRAPRCRAVRSLLRSHYREVLPLATFVRRLGPOGWRLVQRGDP
AAFRALVAQCLVCPWDARPPPAAPSFRQVSCLELVARVLQRL
CERGAENVLAFGFALLDARGGPPPEAFTTSVRSYLPNTVTDALR
GSGAWGLLLRRVGGDDVLVHLLARCALFVLVAPSCAYQVCGPPLY
QLGAATQARPPPHASGPRRRLGCERAWNHSVREAGVPLGLPAPG
ARRRGSASRSLPLPKRPRRGAPEPERTPVGQGSWAHPGRTRG
PSDRGFCVVSAPPAEEATSLEGALSGTRHSHPSVGRQHAGPP
STSRPPRPWDTPCPPVYAETKHFYSSGDKQLRPSFLLSSLRP
SLTGARRLVETIFLGSRPWMPGTPRRLPRLPQRYWQMRPLFLEL
LGNHAQCPYGVLLKTHCPLRAAVTPAAGVCAREKPGQSVAAPEE
EDTDPRLVQLLRQHSSPWQVYGFVRACLRLVPPGLWGSRHNE
RRFLRNTKKFISLGKHAKLSLQELTWKMSVRDCAWLRRSPGVGC
VPAAEHRLREEILAKFLHLMVSVVVELLSFFVYVTTTFQKNR
LFFYRKSVMKLSQSIGIRQHLKRVQLRELSEAENVQHRARPAL
LTSRLRFIPKPDGLRPVNMVYVVGARTFREKRAERLTSRVKA
LFSVLNRYERARRPGLLGASVLGLDDIHRARWTFVLVRVAQDPPP
ELYFVKVDVTGAYDTIPQDRLTEVIASIIKPQNTYCVRRYAVVQ
KAAHGHVRKAFKSHVSTLTDLPYMRQFVAHLQETSPLRDVVI
EQSSSLNEASSGLFDVFLRFMCHHAVRIRGKSYVQCQGIPOGSI
LSTLLCSLCYGDMMENKLFAGIRRDGLLLRLVDDFLLVTPHLTHA
KTLRLTLVRGVPEYGCVMNLKRVVNFVPEDEALGGTAFVQMPA
HGLFPWCGLLLDTRTLEVQSDYSSYARTSIRASLTFNRGFKAGR
NMRRKLFGLVRLKCHSLFLDLQVNSLQTVCTNIYKILLQAYRF
HACVLQLPFHQQVWKNPTFFLRVISDTASLCYSILKAKNAGMSL
GAKGAAGPLPSEAVQWLCHQAFLLKLTRHRVTYVPLGLSLRTAQ
TQLSRKLPGTTLTALEAAANPALPSDFKTILD

FIG. 17

GGCCAAGTTCCTGCACCTGGCTGATGAGTGTACGTCGTCGAGCTGCTCAGGTCTTTCTT
TTATGTACCGGAGACCACGTTTCAAAAGAACAGGCTCTTTTCTACCGGAAGAGTGTCTG
GAGCAAGTTGCAAAGCATGGGAATCAGACAGCACTTGAAGAGGGTGACGCTCGGGGAGCT
GTCGGAAGCAGAGGTCAGGCAGCATCGGGAAGCCAGGCCCGCCCTGCTGACGTCCAGACT
CCGCTTCATCCCCAAGCCTGACGGGCTGCGGCCGATTGTGAACATGGACTACGTCTGTGG
AGCCAGAACGTTCCGAGAGAAAAGAGGGCCGAGCGTCTCACCTCGAGGGTGAAGGCACT
GTTACAGCGTCTCAACTACGAGCGGGCGCGGCCCGCCCGCTCTGGGGCGCTCTGTGCT
GGGCTTGGAGATATCCACAGGGCTTGGCGCACCTTCTGTCTGCGTGTGCGGGCCAGGA
CCCGCCGCTGAGCTGTACTTTGTCAAGGTGATGTGACGGGCGGTACGACACCATCCC
CCAGGACAGGCTCACGGAGGTATCGCCAGCATCATCAAACCCAGAACACGTACTGCGT
GCGTCCGATGCGGTGGTCCAGAAGGCCGCCATGGGCACGTCCCGAAGGCCTTCAAGAG
CCACGTCTCAGTCCAGTGCCAGGGGATCCCGCAGGGCTCCATCCTCTCCACGCTGCTCT
GCAGCCTGTGCTACGGCGACATGGAGAACAAGCTGTTTGGCGGGATTTCGGCGGACGGGC
TGCTCTGCGTCTGGTGGATGATTCTTGTGTGGTGACACCTCACCTCACCCAGCGAAAA
CCTTCCTCAGGACCTGCTCCGAGGTGTCCCTGAGTATGGCTGCGTGGTGAACCTTCCGGA
AGACAGTGGTGAACCTCCCTGTAGAAGACGAGGCCCTGGGTGGCAGGCTTTTGTTCAGA
TGCCGGCCACGGCCTATTCCTGCTGGTGGCGCTGCTGCTGGATACCGGACCTGGAGG
TGCAGAGCGACTACTCCAGCTATGCCCGGACCTCCATCAGAGCCAGTCTCACCTTCAACC
GCGGCTTCAAGGCTGGGAGGAACATGCGTGCAGAACTCTTTGGGGTCTTGGCGCTGAAGT
GTCACAGCCTGTTTCTGGATTGTCAGGTGAACAGCCTCCAGACGGTGTGCACCAACATCT
ACAAGATCCTCCTGCTGAGGCGTACAGGTTTACGCGATGTGTCTGCAGCTCCCATTTT
ATCAGCAAGTTTGAAGAACCACATTTTCTGCGCGTCACTCTGACACGGCTCCC
TCTGCTACTCCATCCTGAAAGCCAGAACGAGGATGTGCTGGGGGCCAAGGGCGCCG
CCGGCC7TCTGCCCCCTCAGGCGCTGACGTGGCTGTGCCACCAAGCATTCCTGCTCAAGC
TGACTCGACACCGTGTACCTACGTGCCACTCCTGGGGTCACTCAGGACAGCCAGACCG
AGCTGAGTCGGAAGCTCCCGGGGACGACGCTGACTGCCCTGGAGGCCGACGCAACCCGG
CACTGCCCTCAGACTTCAAGACCATCTGGACTGATGGCCACCCGCCACAGCCAGGCCG
AGAGCAGACACCAGCAGCCTGTACGCGGGCTCTACGTCCCAGGGAGGGAGGGGGCGGC
CCACACCCAGGCTGACCCGCTGGAGTCTGAGGCTGAGTGAAGTGTGGCCGAGGCT
GCATGTCCGGCTGAAGCTGAGTGTCCGGCTGAGGCTGAGCGAGTGTCCAGCCAGGGC
TGAGTGTCCAGCACACCTGCCGCTTCACTTCCCCACAGGCTGGCGCTCGGCTCCACCCC
AGGGCCAGCTTTTCTCACCAGGAGCCCGCTTCACTCCCCACATAGGAATAGTCCATC
CCCAGATTCCCATTTGTTACCCCTGCGCCCTGCCCTCTTTGCTTCCACCCCAACCATC
CAGGTGGAGACCTGAGAAGGACCTGGGAGCTCTGGGAATTTGAGTGACCAAAGGTGT
GCCCTGTACACAGGCGAGGACCTGCACCTGGATGGGGTCCCTGTGGGTCAAATTGGGG
GGAGGTGCTGTGGGAGTAAATACTGAATATATGAGTTTTCAGTTTGG0AAAAA
AAAAAAAAAAAAA

FIG. 18

23/103

MetSerValTyrValValGluLeuLeuArgSerPhePhe
TyrValThrGluThrThrPheGlnLysAsnArgLeuPhe
PheTyrArgLysSerValTrpSerLysLeuGlnSerIle
GlyIleArgGlnHisLeuLysArgValGlnLeuArgGlu
LeuSerGluAlaGluValArgGlnHisArgGluAlaArg
ProAlaLeuLeuThrSerArgLeuArgPheIleProLys
ProAspGlyLeuArgProIleValAsnMetAspTyrVal
ValGlyAlaArgThrPheArgArgGluLysArgAlaGlu
ArgLeuThrSerArgValLysAlaLeuPheSerValLeu
AsnTyrGluArgAlaArgArgProGlyLeuLeuGlyAla
SerValLeuGlyLeuAspAspIleHisArgAlaTrpArg
ThrPheValLeuArgValArgAlaGlnAspProProPro
GluLeuTyrPheValLysValAspValThrGlyAlaTyr
AspThrIleProGlnAspArgLeuThrGluValIleAla
SerIleIleLysProGlnAsnThrTyrCysValArgArg
TyrAlaValValGlnLysAlaAlaHisGlyHisValArg
LysAlaPheLysSerHisValLeuArgProValProGly
AspProAlaGlyLeuHisProLeuHisAlaAlaLeuGln
ProValLeuArgArgHisGlyGluGlnAlaValCysGly
AspSerAlaGlyArgAlaAlaProAlaPheGlyGly

FIG. 19

GCAGCGCTGCGTCCTGCTGCGCACGTGGGAAGCCCTGGCCCCGCCACCCCCGCG ATG
1
met
10
pro arg ala pro arg cys arg ala val arg ser leu leu arg ser
CCG CGC GCT CCC CGC TGC CGA GCC GTG CGC TCC CTG CTG CGC AGC
20 30
his tyr arg glu val leu pro leu ala thr phe val arg arg leu
CAC TAC CGC GAG GTG CTG CCG CTG GCC ACG TTC GTG CGG CGC CTG
40
gly pro gln gly trp arg leu val gln arg gly asp pro ala ala
GGG CCC CAG GGC TGG CGG CTG GTG CAG CGC GGG GAC CCG GCG GCT
50 60
phe arg ala leu val ala gln cys leu val cys val pro trp asp
TTC CGC GCG CTG GTG GCC CAG TGC CTG GTG TGC GTG CCC TGG GAC
70
ala arg pro pro pro ala ala pro ser phe arg gln val ser cys
GCA CGG CCG CCC CCC GCC GCC CCC TCC TTC CGC CAG GTG TCC TGC
80 90
leu lys glu leu val ala arg val leu gln arg leu cys glu arg
CTG AAG GAG CTG GTG GCC CGA GTG CTG CAG AGG CTG TGC GAG CGC
100
gly ala lys asn val leu ala phe gly phe ala leu leu asp gly
GGC GCG AAG AAC GTG CTG GCC TTC GGC TTC GCG CTG CTG GAC GGG
110 120
ala arg gly gly pro pro glu ala phe thr thr ser val arg ser
GCC CGC GGG GGC CCC CCC GAG GCC TTC ACC ACC AGC GTG CGC AGC

FIG. 20

24/103

```

                                130
tyr leu pro asn thr val thr asp ala leu arg gly ser gly ala
TAC CTG CCC AAC ACG GTG ACC GAC GCA CTG CCG GGG AGC GGG GCG

                                140
trp gly leu leu leu arg arg val gly asp asp val leu val his
TGG GGG CTG CTG CTG CGC CGC GTG GGC GAC GAC GTG CTG GTT CAC

                                150
leu leu ala arg cys ala leu phe val leu val ala pro ser cys
CTG CTG GCA CGC TGC GCG CTC TTT GTG CTG GTG GCT CCC AGC TGC

                                160
ala tyr gln val cys gly pro pro leu tyr gln leu gly ala ala
GCC TAC CAG GTG TGC GGG CCG CCG CTG TAC CAG CTC GGC GCT GCC

                                170
thr gln ala arg pro pro pro his ala ser gly pro arg arg arg
ACT CAG GCC CGG CCC CCG CCA CAC GCT AGT GGA CCC CGA AGG CGT

                                180
leu gly cys glu arg ala trp asn his ser val arg glu ala gly
CTG GGA TGC GAA CGG GCC TGG AAC CAT AGC GTC AGG GAG GCC GGG

                                190
val pro leu gly leu pro ala pro gly ala arg arg arg gly gly
GTC CCC CTG GGC CTG CCA GCC CCG GGT GCG AGG AGG CGC GGG GGC

                                200
ser ala ser arg ser leu pro leu pro lys arg pro arg arg gly
AGT GCC AGC CGA AGT CTG CCG TTG CCC AAG AGG CCC AGG CGT GGC

                                210
ala ala pro glu pro glu arg thr pro val gly gln gly ser trp
GCT GCC CCT GAG CCG GAG CGG ACG CCC GTT GGG CAG GGG TCC TGG

                                220
ala his pro gly arg thr arg gly pro ser asp arg gly phe cys
GCC CAC CCG GGC AGG ACG CGT GGA CCG AGT GAC CGT GGT TTC TGT

                                230
val val ser pro ala arg pro ala glu glu ala thr ser leu glu
GTG GTG TCA CCT GCC AGA CCC GCC GAA GAA GCC ACC TCT TTG GAG

                                240
gly ala leu ser gly thr arg his ser his pro ser val gly arg
GGT GCG CTC TCT GGC ACG CGC CAC TCC CAC CCA TCC GTG GGC CGC

                                250
gln his his ala gly pro pro ser thr ser arg pro pro arg pro
CAG CAC CAC GCG GGC CCC CCA TCC ACA TCG CGG CCA CCA CGT CCC

                                260
trp asp thr pro cys pro pro val tyr ala glu thr lys his phe
TGG GAC ACG CCT TGT CCC CCG GTG TAC GCC GAG ACC AAG CAC TTC

                                270
                                280
                                290
                                300
                                310
                                320
                                330

```

FIG. 20
(CONTINUED)

25/103

```

                                340
leu tyr ser ser gly asp lys glu gln leu arg pro ser phe leu
CTC TAC TCC TCA GGC GAC AAG GAG CAG CTG CGG CCC TCC TTC CTA

                                350                                360
leu ser ser leu arg pro ser leu thr gly ala arg arg leu val
CTC AGC TCT CTG AGG CCC AGC CTG ACT GGC GCT CGG AGG CTC GTG

                                370
glu thr ile phe leu gly ser arg pro trp met pro gly thr pro
GAG ACC ATC TTT CTG GGT TCC AGG CCC TGG ATG CCA GGG ACT CCC

                                380                                390
arg arg leu pro arg leu pro gln arg tyr trp gln met arg pro
CGC AGG TTG CCC CGC CTG CCC CAG CGC TAC TGG CAA ATG CGG CCC

                                400
leu phe leu glu leu leu gly asn his ala gln cys pro tyr gly
CTG TTT CTG GAG CTG CTT GGG AAC CAC GCG CAG TGC CCC TAC GGG

                                410                                420
val leu leu lys thr his cys pro leu arg ala ala val thr pro
GTG CTC CTC AAG ACG CAC TGC CCG CTG CGA GCT GCG GTC ACC CCA

                                430
ala ala gly val cys ala arg glu lys pro gln gly ser val ala
GCA GCC GGT GTC TGT GCC CGG GAG AAG CCC CAG GGC TCT GTG GCG

                                440                                450
ala pro glu glu glu asp thr asp pro arg arg leu val gln leu
GCC CCC GAG GAG GAG GAC ACA GAC CCC CGT CGC CTG GTG CAG CTG

                                460
leu arg gln his ser ser pro trp gln val tyr gly phe val arg
CTC CGC CAG CAC AGC AGC CCC TGG CAG GTG TAC GGC TTC GTG CGG

                                470                                480
ala cys leu arg arg leu val pro pro gly leu trp gly ser arg
GCC TGC CTG CGC CGG CTG GTG CCC CCA GGC CTC TGG GGC TCC AGG

                                490
his asn glu arg arg phe leu arg asn thr lys lys phe ile ser
CAC AAC GAA CGC CGC TTC CTC AGG AAC ACC AAG AAG TTC ATC TCC

                                500                                510
leu gly lys his ala lys leu ser leu gln glu leu thr trp lys
CTG GGG AAG CAT GCC AAG CTC TCG CTG CAG GAG CTG ACG TGG AAG

                                520
met ser val arg asp cys ala trp leu arg arg ser pro gly val
ATG AGC GTG CGG GAC TGC GCT TGG CTG CGC AGG AGC CCA GGG GTT

                                530                                540
gly cys val pro ala ala glu his arg leu arg glu glu ile leu
GGC TGT GTT CCG GCC GCA GAG CAC CGT CTG CGT GAG GAG ATC CTG

```

FIG. 20
(CONTINUED)

26/103

550
ala lys phe leu his trp leu met ser val tyr val val glu leu
GCC AAG TTC CTG CAC TGG CTG ATG AGT GTG TAC GTC GTC GAG CTG

560 570
leu arg ser phe phe tyr val thr glu thr thr phe gln lys asn
CTC AGG TCT TTC TTT TAT GTC ACG GAG ACC ACG TTT CAA AAG AAC

580
arg leu phe phe tyr arg lys ser val trp ser lys leu gln ser
AGG CTC TTT TTC TAC CGG AAG AGT GTC TGG AGC AAG TTG CAA AGC

590 600
ile gly ile arg gln his leu lys arg val gln leu arg glu leu
ATT GGA ATC AGA CAG CAC TTG AAG AGG GTG CAG CTG CGG GAG CTG

610
ser glu ala glu val arg gln his arg glu ala arg pro ala leu
TCG GAA GCA GAG GTC AGG CAG CAT CGG GAA GCC AGG CCC GCC CTG

620 630
leu thr ser arg leu arg phe ile pro lys pro asp gly leu arg
CTG ACG TCC AGA CTC CGC TTC ATC CCC AAG CCT GAC GGG CTG CGG

640
pro ile val asn met asp tyr val val gly ala arg thr phe arg
CCG ATT GTG AAC ATG GAC TAC GTC GTG GGA GCC AGA ACG TTC CGC

650 660
arg glu lys arg ala glu arg leu thr ser arg val lys ala leu
AGA GAA AAG AGG GCC GAG CGT CTC ACC TCG AGG GTG AAG GCA CTG

670
phe ser val leu asn tyr glu arg ala arg arg pro gly leu leu
TTC AGC GTG CTC AAC TAC GAG CGG GCG CGG CGC CCC GGC CTC CTG

680 690
gly ala ser val leu gly leu asp asp ile his arg ala trp arg
GGC GCC TCT GTG CTG GGC CTG GAC GAT ATC CAC AGG GCC TGG CGC

700
thr phe val leu arg val arg ala gln asp pro pro pro glu leu
ACC TTC GTG CTG CGT GTG CGG GCC CAG GAC CCG CCG CCT GAG CTG

710 720
tyr phe val lys val asp val thr gly ala tyr asp thr ile pro
TAC TTT GTC AAG GTG GAT GTG ACG GGC GCG TAC GAC ACC ATC CCC

730
gln asp arg leu thr glu val ile ala ser ile ile lys pro gln
CAG GAC AGG CTC ACG GAG GTC ATC GCC AGC ATC ATC AAA CCC CAG

740 750
asn thr tyr cys val arg arg tyr ala val val gln lys ala ala
AAC ACG TAC TGC GTG CGT CGG TAT GCC GTG GTC CAG AAG GCC GCC

FIG. 20
(CONTINUED)

27/103

760
his gly his val arg lys ala phe lys ser his val leu arg pro
CAT GGG CAC GTC CGC AAG GCC TTC AAG AGC CAC GTC CTA CGT CCA

770 780
val pro gly asp pro ala gly leu his pro leu his ala ala leu
GTG CCA GGG GAT CCC GCA GGG CTC CAT CCT CTC CAC GCT GCT CTG

790
gln pro val leu arg arg his gly glu gln ala val cys gly asp
CAG CCT GTG CTA CGG CGA CAT GGA GAA CAA GCT GTT TGC GGG GAT

800 807
ser ala gly arg ala ala pro ala phe gly gly OP
TCG GCG GGA CGG GCT GCT CCT GCG TTT GGT GGA TGA TTCTTGTTGGT

GACACCTCACCTCACCCACGCGAAAACCTTCCTCAGGACCCTGGTCCGAGGTGTCCCTGA
GTATGGCTGCGTGGTGAACCTTGCGGAAGACAGTGGTGAACCTCCCTGTAGAAGACGAGGC
CCTGGGTGGCAGGGCTTTTGTTCAGATGCCGCCCCACGGCCTATTCCCCTGGTGCGGCCT
GCTGCTGGATAACCGGACCCTGGAGGTGCAGAGCGACTACTCCAGCTATGCCCGGACCTC
CATCAGAGCCAGTCTCACCTTCAACCGCGGCTTCAAGGCTGGGAGGAACATGCGTCGCAA
ACTCTTTGGGGTCTTGCGGCTGAAGTGTACAGCCTGTTTCTGGATTTGCAGGTGAACAG
CCTCCAGACGGTGTGCACCAACATCTACAAGATCCTCCTGCTGCAGGCGTACAGGTTTCA
CGCATGTGTGCTGCAGCTCCCATTTCATCAGCAAGTTTGAAGAACCCACATTTTCTCT
GCGCGTCATCTCTGACACGGCCTCCCTCTGCTACTCCATCCTGAAAGCCAAGAACGCAGG
GATGTGCTGCGTGGGGGCCAAGGGCGCCGCCGCTCTGCCCTCCGAGGCCGTGCAGTGGCT
GTGCCACCAAGCATTCTCTGCTCAAGCTGACTCGACACCGTGTACCTACGTGCCACTCCT
GGGGTCACTCAGGACAGCCAGACGCGAGCTGAGTCGGAAGCTCCCGGGGACGAGCTGAC
TGCCCTGGAGGCCGAGCCAACCCGGCACTGCCCTCAGACTTCAAGACCATCCTGGACTG
ATGGCCACCCGCCACAGCCAGGCCGAGAGCAGACACCAGCAGCCCTGTACGCGGGGCT
CTACGTCCCAGGGAGGGAGGGGCGGCCACACCCAGGCCCGCACCGCTGGGAGTCTGAGG
CCTGAGTGAGTGTGTTGGCCGAGGCCGTGCATGTCCGGCTGAAGGCTGAGTGTCCGGCTGAG
GCCTGAGCGAGTGTCCAGCCAAGGGCTGAGTGTCCAGCACACCTGCCGTCTTCACTTCCC
CACAGGCTGGCGCTCGGCTCCACCCAGGGCCAGCTTTTCTCACCAGGAGCCCGGCTTC
CACTCCCCACATAGGAATAGTCCATCCCCAGATTGCGCATTTGTTACCCCTCGCCCTGCC
CTCCTTTGCCTTCCACCCCAACCATCCAGGTGGAGACCCTGAGAAGGACCCCTGGGAGCTC
TGGAATTTGGAGTGACCAAGGTGTGCCCTGTACACAGGCGAGGACCCTGCACCTGGAT
GGGGTCCCTGTGGGTCAAATTGGGGGAGGTGCTGTGGGAGTAAATACTGAATATATG
AGTTTTTCAGTTTTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

FIG. 20
(CONTINUED)

28/103

1 CCATGGGACCCACTGCAGGGGAGCTGGGAGGCTGCAGGCTTCAGGTCCAGTGGGGTTG
GGTACCCTGGGTGACGTCCCCGTCGACCCTCCGACGTCCGAAGTCCAGGGTCACCCCAAC

61 CCATCTGCCAGTAGAAACCTGATGTAGAATCAGGGCGGAGTGTGGACACTGTCTGAAAT
GGTAGACGGTCATCTTTGGACTACATCTTAGTCCCGCGCTCACACCTGTGACAGGACTTA

121 CTCAATGTCTCAGTGTGTGCTGAAACATGTAGAAATTAAAGTCCATCCCTCTACTCTAC
GAGTTACAGAGTCACACACGACTTTGTACATCTTTAATTTTCAGGTAGGAGGATGAGATG

181 TGGGATTGAGCCCCCTTCCCTATCCCCCCCCAGGGGCGAGGAGTTCTCTCACTCTCTGTG
ACCCTAACCTCGGGGAAGGGATAGGGGGGGGTCCCCGTCTCTCAAGGAGAGTGAGGACAC

241 GAGGAAGGAATGATACTTTGTTATTTTCACTGCTGGTACTGAATCCACTGTTTCATTGT
CTCCTTCTCTTACTATGAAACAATAAAAAGTGACGACCATGACTTAGGTGACAAAGTAAAC

301 TTGGTTTGTTTGTTTGTGTTTGGAGAGGCGGTTTCACTCTTGTGCTCAGGCTGGAGGGAG
AACCACAAACAAACAAACAAACCTCTCCGCCAAAGTGAGAACAAACGAGTCCGACCTCCCTC

361 TGCAATGGCGCGATCTTGGCTTACTGCAGCCTCTGCCTCCAGGTTCAAGTGATTCTCTCT
ACGTTACCGCGCTAGAACCGAATGACGTCCGAGACGGAGGGTCCAAGTTCACTAAGAGGA

421 GCTTCCGCCTCCCATTTGGCTGGGATTACAGGCACCCGCCACCATGCCCAGCTAAATTTT
CGAAGGCGGAGGGTAAACCGACCCTAATGTCCGTGGGCGGTGGTACGGGTTCGATTAAAAA

481 TGTATTTTGTAGAGACGGGGGTGGGGGTGGGGTTTCAACATGTTGGCCAGGCTGGTCTC
ACATAAAAAATCATCTCTGCCCCACCCCCACCCAAGTGGTACAACCGGTCCGACCAGAG

541 GAACTTCTGACCTCAGATGATCCACCTGCCTCTGCCTCCTAAAGTGCTGGGATTACAGGT
CTTGAAGACTGGAGTCTACTAGGTGGACGGAGACGGAGGATTTCACGACCCTAATGTCCA

601 GTGAGCCACCATGCCCAGCTCAGAATTTACTCTGTTTAGAAACATCTGGGTCTGAGGTAG
CACTCGGTGGTACGGGTGAGTCTTAAATGAGACAAATCTTTGTAGACCCAGACTCCATC

661 GAAGCTCACCCCACTCAAGTGTGTGGTGTTTAAGCCAATGATAGAATTTTATTGT
CTTCGAGTGGGGTGAGTTCACAACACCACAAATTCGGTTACTATCTTAAAAAATAACA

721 TGTTAGAACACTCTTGATGTTTTACACTGTGATGACTAAGACATCATCAGCTTTTCAAAG
ACAATCTTGTGAGAACTACAAAATGTGACACTACTGATTCTGTAGTAGTCGAAAAGTTTC

FIG. 21

29/103

CAP

*****>

781 ACACACTAACTGCACCCATAATACTGGGGTGTCTTCTGGGTATCAGCGATCTTCATTGAA
TGTGTGATTGACGTGGGTATTATGACCCACAGAAGACCCATAGTCGCTAGAAGTAACCT

CAP

*****>

841 TGCCGGGAGGCGTTTCTCGCCATGCACATGGTGTAAATTACTCCAGCATAATCTTCTGC
ACGGCCCTCCGCAAAGGAGCGGTACGTGTACCACAAATTAATGAGGTCGTATTAGAAGACG

***>

901 TTCCATTTCTTCTCTTCCCTCTTTTAAATTTGTGTTTTCTATGTTGGCTTCTCTGCAGAG
AAGGTAAAGAAGAGAAGGGAGAAAATTTTAAACAAAAAGATACAACCGAAGAGACGTCTC

CAP

*****>

961 AACCAGTGTAAGCTACAACCTTAACTTTTGTGGAACAAATTTTCCAAACCGCCCTTTGC
TTGGTCACATTCGATGTTGAATTGAAAACAACCTTGTTTAAAAGGTTTGGCGGGGAAACG

1021 CCTAGTGGCAGAGACAATTACAAACACAGCCCTTTAAAAGGCTTAGGGATCACTAAGG
GGATCACCGTCTCTGTAAAGTGTGTTGTGTCGGGAAATTTTCCGAATCCCTAGTGATTCC

1081 GGATTTCTAGAAGAGCGACCCGTAATCCTTAAGTATTTACAAGACGAGGCTAACCTCCAG
CCTAAAGATCTTCTCGCTGGGCATTAGGAATTCATAAATGTTCTGCTCCGATTGGAGGTC

1141 CGAGCGTGACAGCCAGGGAGGGTGCGAGGCTGTTCAAATGCTAAGCTTCCATAAATAA
GCTCGCACTGTGGGTCCCTCCACGCTCCGACAAGTTTACGATTCCAAGGTATTTATT

1201 AGCAAATTTCTCCGGCAGTTTCTGGAAAGTAGGAAAGGTTAACATTTAAGGTTGCGTTT
TCGTTTAAAGGAGGCCGTCAAAGACCTTTTCATCCTTTCCAATTGTAAATTTCAACGCAAA

1261 GTTAGCATTTCACTGTTTGGCGACCTCAGCTAACAGCATCCCTGCAAGGCCCTCGGGAGAC
CAATCGTAAAGTCACAAACGCTGGAGTCGATTGTCTGTTAGGGACGTTCCGGAGCCCTCTG

1321 CCAGAAGTTTCTCGCCCTTAGATCCAACTTGAGCAACCCGGAGTCTGGATTCTCTGGGA
GGTCTTCAAAGAGCGGGGAATCTAGGTTTGAACCTCGTTGGGCCTCAGACCTAAGGACCTT

TopoII

*****>

1381 AGTCCTCAGCTGTCCTGCGGTTGTGCCGGGGCCCCAGGTCTGGAGGGGACCAGTGGCCGT
TCAGGAGTCGACAGGACGCCAACACGGCCCCGGGGTCCAGACCTCCCTGGTCACCGGCA

1441 GTGGCTTCTACTGCTGGGCTGGAAGTCGGGCCTCCTAGCTCTGCAGTCCGAGGCTTGGAG
CACCGAAGATGACGACCCGACCTTCAGCCGGAGGATCGAGACGTCAAGCTCCGAACCTC

1501 CCAGGTGCCTGGACCCCGAGGCTGCCCTCCACCCTGTGCGGGCGGGATGTGACCAGATGT
GGTCCACGGACCTGGGGCTCCGACGGGAGGTGGGACACGCCCCCTACACTGGTCTACA

1561 TGGCCTCATCTGCCAGACAGAGTGCCGGGGCCAGGGTCAAGGCCGTTGTGGCTGGTGTG
ACCGGAGTAGACGGTCTGTCTACGGCCCCGGGTCCAGTTCCGGCAACACCGACCACAC

1621 AGGCGCCCCGTTGCGCGCCAGCAGGAGCGCCTGGCTCCATTTCCACCCTTTCTCGACGG
TCCGCGGGCCACGCGCCGTCGTCTCGCGGACCGAGGTAAAGGTTGGGAAAGAGCTGCC

FIG. 21
(CONTINUED)

30/103

1681 GACCGCCCCGGTGGGTGATTAACAGATATTGGGGTGGTTTGCTCATGGTGGGGACCCCTT
CTGGCGGGGCCACCCACTAATTGTCTATAACCCACCAAACGAGTACCACCCCTGGGGAA

1741 CGCCGCCTGAGAACCTGCAAAGAGAAATGACGGGCCTGTGTCAAGGAGCCCAAGTCGCGG
GCGGCGGACTCTTGGACGTTTCTCTTTACTGCCCGGACACAGTTCCTCGGGTTCAGCGCC

1801 GGAAGTGTTCAGGGAGGCACTCCGGGAGGTCCCGGTGCCCGTCCAGGGAGCAATGCGT
CCTTACAACGTCCCTCCGTGAGGCCCTCCAGGGCGCACGGGCAGGTCCCTCGTTACGCA

1861 CCTCGGGTTCGTCCCCAGCCGCGTCTACGCGCCTCCGTCCCTCCCTTCACGTCCGGCATT
GGAGCCCAAGCAGGGGTCGGCGCAGATGCCGCGAGGCAGGAGGGGAAGTGCAGGCCGTAA

1921 CGTGGTGCCCGGAGCCCGACGCCCCGCGTCCGGACCTGGAGGCAGCCCTGGGTCTCCGGA
GCACCACGGGCCTCGGGCTGCGGGGCGCAGGCCTGGACCTCCGTCCGGACCCAGAGGCCT

1981 TCAGGCCAGCGGCCAAAGGGTCGCCGCACGCACCTGTTCCAGGGCCTCCACATCATGGC
AGTCCGGTCGCCGGTTTCCAGCGGCGTGCCTGGACAAGGGTCCCGGAGGTGTAGTACCG

2041 CCCTCCCTCGGGTTACCCACAGCCTAGGCCGATTTCGACCTCTCTCGCTGGGGCCCTCG
GGGAGGGAGCCCAATGGGGTGTGCGATCCGGCTAAGCTGGAGAGAGGCGACCCCGGGAGC

Sp1

2101 CTGGCGTCCCTGCACCCCTGGGAGCGCGAGCGGCGCGGGCGGGGAAGCGCGGCCAGAC
GACCGCAGGACGTGGGACCTCGCGCTCGCCGCGCGCCCGCCCTTCGCGCGGGTCTG

2161 CCCC GGTTCCGCGGAGCAGCTGCGCTGTGCGGGCCAGGCCGGGCTCCCACTGGATTG
GGGGCCAGCGGGGCTCTGCGACGCGACAGCCCGGTCCGGCCGAGGGTACCTAAGC

2221 CGGGCAACAGACGCCCAGGACCGCGCTTCCACGTGGCGGAGGGACTGGGGACCCGGGCA
GCCCGTTGTCTGCGGGTCTTGGCGCGAAGGGTGCACCGCCTCCCTGACCCCTGGGCCCGT

Sp1
=====

E2F

2281 CCGGTCTGCCCCCTTACCTTCCAGCTCCGCTCGTCCGCGCGGAACCCCGCCCGTCCC
GGCCAGGACGGGGAAGTGAAGGTGAGGCGGAGCAGGCGCGCCTTGGGGCGGGCAGGG

2341 GAACCTTCCCGGGTCCCGGCCCCAGCCCTTCCGGGCCATCCAGCCGTCCTCGTTCT
CTTGGGAAGGGCCCAGGGCGCGGTTCGGGAAGGCCCGGTAGGGTCCGGCAGGGCAAGGA

Sp1
=====

E2F

NFkB

2401 TTTCGCGGCCCCCGCCCTCTCCTCGCGGCGCGAGTTTCAGGCAGCGCTGCGTCTGCTGC
AAAGGCGCCGGGGCGGAGAGGAGCGCCGCGCTCAAAGTCCGTGCGGACGCAGGACGACG

hTRT5'
*****>

2461 GCACGTGGGAAGCCCTGGCCCCGGCCACCCCGCGATGCCGCGCGCTCCCCGCTGCCGAG
CGTGACCCCTTCGGGACCGGGGCGGTGGGGGCGTACGGCGCGCGAGGGGCGACGGCTC

2521 CCGTGCGCTCCCTGCTGCGCAGCCACTACCGCGAGGTGCTGCCGCTGGCCACGTTCTGTGC
GGCAGCGAGGGACGACCGTCCGGTGATGGCGCTCCACGACGGCGACCGGTGCAAGCACG

FIG. 21
(CONTINUED)

31/103

E2F

2581 GGGCCTGGGGCCCCAGGGCTGGCGGCTGGTGCAGCGCGGGGACCGGGCGGCTTTCCGCG
CCGCGGACCCCGGGTCCCGACCGCCGACCACGTCGCGCCCTGGGCGCGCGAAAGCGC

*

2641 CGCTGGTGGCCAGTGCCTGGTGTGCGTGCCCTGGGACGCACGGCGCCCCCGCCGCCC
CGCACCACCGGGTCACGGACCACACGCACGGGACCCTGCGTGCCGGCGGGGGCGCGGG

NFkB

=====

2701 CCTCCTTCCGCCAGGTGGGCTTCCCCGGGGTGGCGTCCGGCTGGGGTTGAGGGCGGCCG
GGAGGAAGGCGGTCCACCCGAGGGGCCCCAGCCGAGGCCGACCCCAACTCCGCCGGC

Topo_II_cleavag

:::::::::::::

NFkB

+++++++

NFkB

=====

Intron1

*****>

2761 GGGGGAACCAGCGACATGCGGAGAGCAGCGCAGGCGACTCAGGGCGCTTCCCCCGCAGGT
CCCCCTTGGTCGCTGTACGCCTCTCGTTCGCTCCGCTGAGTCCCGCAAGGGGGCGTCCA

e_site

::::

2821 GTCCTGCCTGAAGGAGCTGGTGGCCCGAGTGTGTCAGAGGCTGTGCGAGCGCGGCGCGAA
CAGGACGGACTTCCTCGACCACCGGGCTCACGACGTCCTCGACACGCTCGCGCCGCGCTT

2881 GAACGTGCTGGCCTTTCGGCTTCGCGCTGCTGGACGGGGCCCGGGGGCCCCCGAGGC
CTTGACGACCGGAAGCCGAAGCGCGACGACCTGCCCGGGCGCCCCGGGGGGCTCCG

2941 CTTCAACACCAGCGTGCAGCTACCTGCCCAACACGGTGACCGACGCACTGCGGGGGAG
GAAGTGGTGGTGCACGCGTCGATGGACGGGTTGTGCCACTGGCTGCGTGACGCCCCCTC

3001 CGGGCGTGGGGCTGTGCTGCGCCGCGTGGGCGACGACGTGCTGGTTACCTGCTGGC
GCCCCGACCCCCGACGACGCGGCGCACCCGCTGCTGCACGACCAAGTGGACGACCG

3061 ACGCTGCGCGCTCTTTGTGCTGGTGGCTCCAGCTGCGCCTACCAGGTGTGCGGGCCGCC
TGCAGCGCGGAGAAACACGACCACCGAGGGTTCGACGCGGATGGTCCACACGCCCGGCGG

3121 GCTGTACCAGCTCGGCGCTGCCACTCAGGCCCGGCCCCCGCCACACGCTAGTGGACCCG
CGACATGGTTCGAGCCGCGACGGTGAGTCCGGGCGGGGGCGGTGTGCGATCACCTGGGGC

3181 AAGGCGTCTGGGATGCGAACGGGCCTGGAACCATAGCGTCAGGGAGGCCGGGGTCCCCCT
TTCCGCAGACCTTACGCTTGCCCGGACCTTGGTATCGCAGTCCCTCCGGCCCCAGGGGA

3241 GGGCCTGCCAGCCCCGGGTGCGAGGAGGCGGGGGCAGTGCCAGCCGAAGTCTGCCGTT
CCCGGACGGTCGGGGCCACGCTCTCCGCGCCCCGTCACGGTCGGCTTCAGACGGCAA

3301 GCCCAAGAGGCCCCAGGCGTGGCGCTGCCCTGAGCCGAGCGGACGCCCGTTGGGCAGGG
CGGGTTCTCCGGGTCCGACCGCGACGGGACTCGGCCTCGCCTCGGGCAACCCGTCCC

FIG. 21
(CONTINUED)

32/103

3361 GTCCTGGGCCCCACCCGGGCAGGACGCGTGACCGAGTGACCGTGGTTTCTGTGTGGTGTGTC
CAGGACCCGGGTGGGCCCCGTCTGCGCACCTGGCTCACTGGCACCAAAGACACACCACAG

3421 ACCTGCCAGACCCGCCGAAGAAGCCACCTCTTTGGAGGGTGGCTCTCTGGCACGCGCCA
TGGACGGTCTGGGCGGCTTCTTCGGTGGAGAAACCTCCACGCGAGAGACCGTGGCGGGT

3481 CTCCCACCCATCCGTGGGCGGCCAGCACCACGCGGGCCCCCATCCACATCGCGGCCACC
GAGGGTGGGTAGGCACCCGGCGGTCTGTGGTGGCCCCGGGGGTAGGTGTAGCGCCGGTGG

3541 ACGTCCCTGGGACACGCCCTTGTCCCCCGGTGTACGCGAGACCAAGCACTTCTCTACTC
TGCAGGGACCTGTGCGGAACAGGGGGCCACATGCGGCTCTGGTTCTGTGAAGGAGATGAG

3601 CTCAGGCGACAAGGAGCAGCTGCGGCCCTCTTCTCTACTCAGCTCTCTGAGGCCAGCCT
GAGTCCGCTGTCTCTCGTCGACGCCGGGAGGAAGGATGAGTCGAGAGACTCCGGGTCCGA

3661 GACTGGCGCTCGGAGGCTCGTGGAGACCATCTTTCTGGGTTCAGGCCCTGGATGCCAGG
CTGACCGCGAGCCTCCGAGCACCTCTGGTAGAAAGACCCAAGGTCCGGGACCTACGGTCC

3721 GACTCCCCCGAGGTTGCCCCGCTGCCCCAGCGCTACTGGCAAATGCGGGCCCTGTTTCT
CTGAGGGCGTCCAACGGGGCGGACGGGGTGGCGATGACCGTTTACGCCGGGGACAAAGA

3781 GGAGCTGCTTGGGAACCACGCGCAGTGCCCCCTACGGGGTGCTCTCAAGACGCACTGCC
CCTCGACGAACCCCTTGGTGGCGCTCACGGGGATGCCCCACGAGGAGTTCTGGCTGACGGG

3841 GCTGCGAGCTGCGGTACCCCCAGCAGCCGGTGTCTGTGCCCCGGGAGAAGCCCCAGGGCTC
CGACGCTCGACGCCAGTGGGGTCTGCGGCCACAGACACGGGCCCTCTTCGGGGTCCCGAG

3901 TGTGGCGGCCCCCGAGGAGGAGGACACAGACCCCCGTGCGCTGGTGCAGCTGCTCCGCCA
ACACCGCCGGGGCTCTCTCTCTGTGTCTGGGGGACGCGACACAGTCCGACGAGGCGGT

3961 GCACAGCAGCCCCTGGCAGGTGTACGGCTTCGTGCGGGCCTGCCTGCGCCGGCTGGTGCC
CGTGTCTGTCGGGGACCGTCCACATGCCGAAGCACGCCCGGACGGACGCGGCCGACACCG

4021 CCCAGGCCTCTGGGGCTCCAGGCACAACGAACGCCGCTTCTCTCAGGAACACCAAGAAGTT
GGGTCCGGAGACCCCGAGGTCCGTGTGCTTGCGGCGAAGGAGTCTTGTGGTTCTTCAA

4081 CATCTCCCTGGGGAAGCATGCCAAGCTCTCGCTGCAGGAGCTGACGTGGAAGATGAGCGT
GTAGAGGGACCCCTTCGTACGGTTCGAGAGCGACGTCTCTGACTGCACCTTCTACTCGCA

4141 GCGGGACTGCGCTTGGCTGCGCAGGAGCCCAGGTGAGGAGGTGGTGGCCGTGAGGGCCC
CGCCCTGACGCGAACCAGCGCTCTCGGGTCCACTCTCCACCACCGGCAGCTCCCGGG

Intron2

4201 AGGCCCCAGAGCTGAATGCAGTAGGGGCTCAGAAAAGGGGGCAGGCAGAGCCCTGGTCCT
TCCGGGGTCTCGACTTACGTATCCCGAGTCTTTTCCCCCGTCCGTCTCGGGACCAGGA

4261 CCTGTCTCCATCGTCAGTGCGGCACAGTGCGCTTTTTCGCTCAGGACGTGAGTGACACG
GGACAGAGGTAGCAGTGCACCCGTGTGCACCGAAAAGCGAGTCTCTCAGCTCACCTGTGC

*****>

4321 GTGATCGAGGTGCAC
CACTAGCTCCAGCTG

FIG. 21
(CONTINUED)

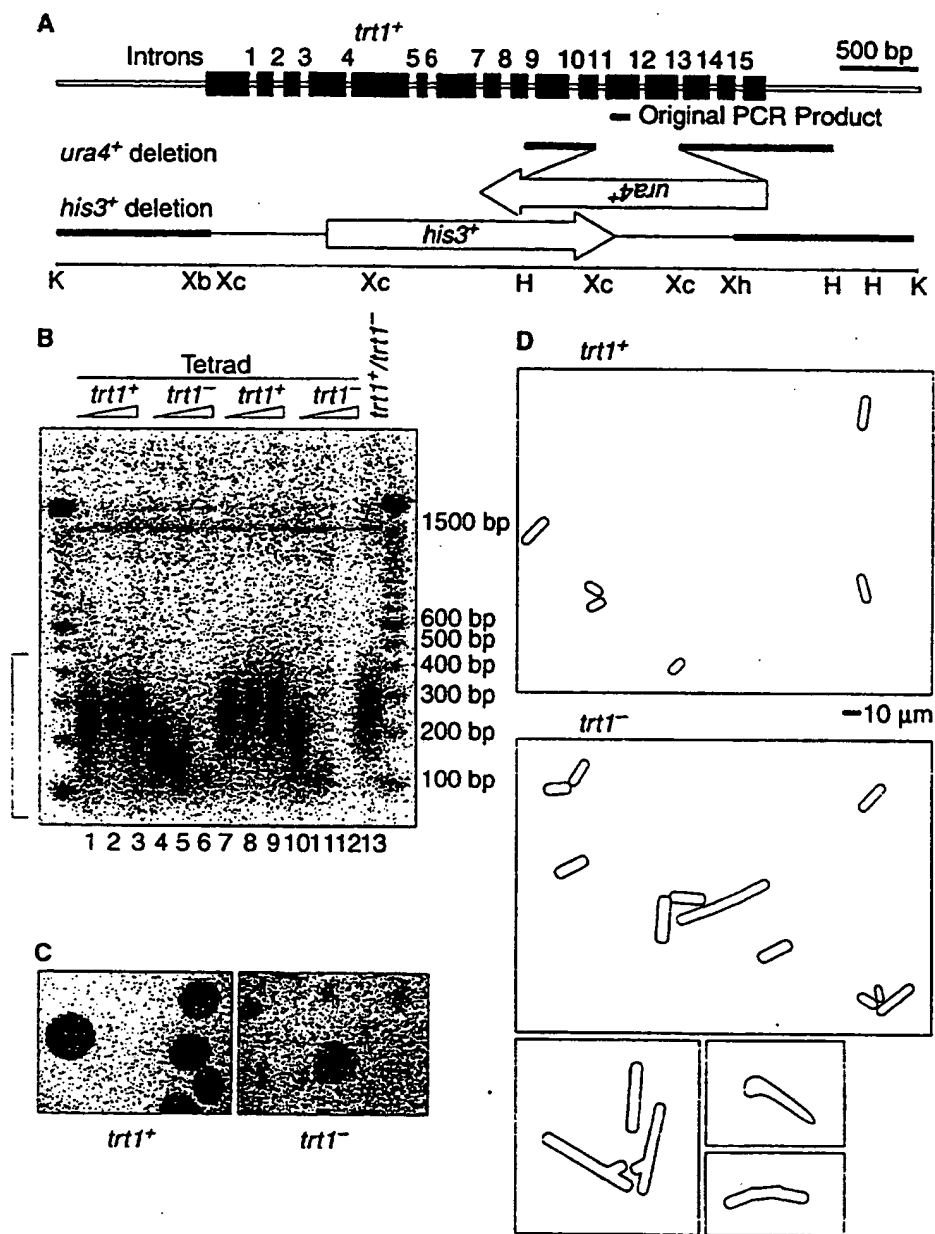


FIG. 22

34/103

gccaagttcctgcactggctgatgagtgtgtacgtcgctcgagctgctcaggtctttcttt
tatgtcacggagaccacgtttcaaaagaacaggctctttttctaccggaagagtgtctgg
agcaagttgcaaagcattggaatcagacagcacttgaagaggggtgcagctgcgggacgtg
tcggaagcagaggtcaggcagcatcggggaagccaggcccgccctgctgacgtccagactc
cgcttcaccccccaagcctgacgggctgcgccgattgtgaacatggactacgtcgtggga
gccagaacgttccgcagagaaaagaggccgagcgtctcacctcgaggggtgaaggcactg
ttcagcgtgctcaactacgagcgggcgcg

FIG. 23

TCTACCTTGACAGACCTCCAGCCGTACATGCGACAGTTCGTGGCTCACCTGCAGGAG
ACCAGCCCGCTGAGGGATGCCGTCGTCATCGAGCAGAGCTCCTCCCTGAATGAGGCC
AGCAGTGGCCTCTTCGACGCTTCTCTACGCTTCATGTGCCACCACGCCGTGCGCATC
AGGGGCAAGTC

FIG. 24

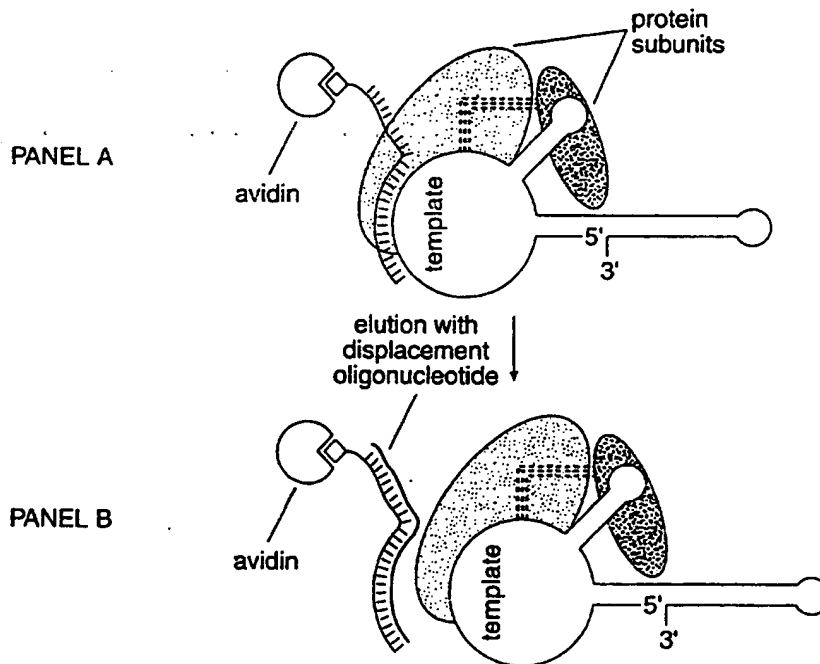
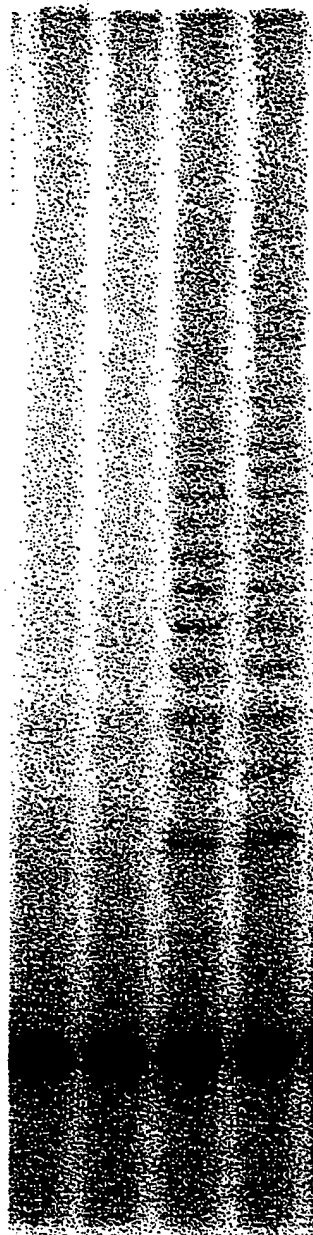


FIG. 26

35/103

pBB5212 pGRN133



← Internal Control

Approximate Cell No. 5,000 5,000 5,000 5,000

FIG. 25

36/103

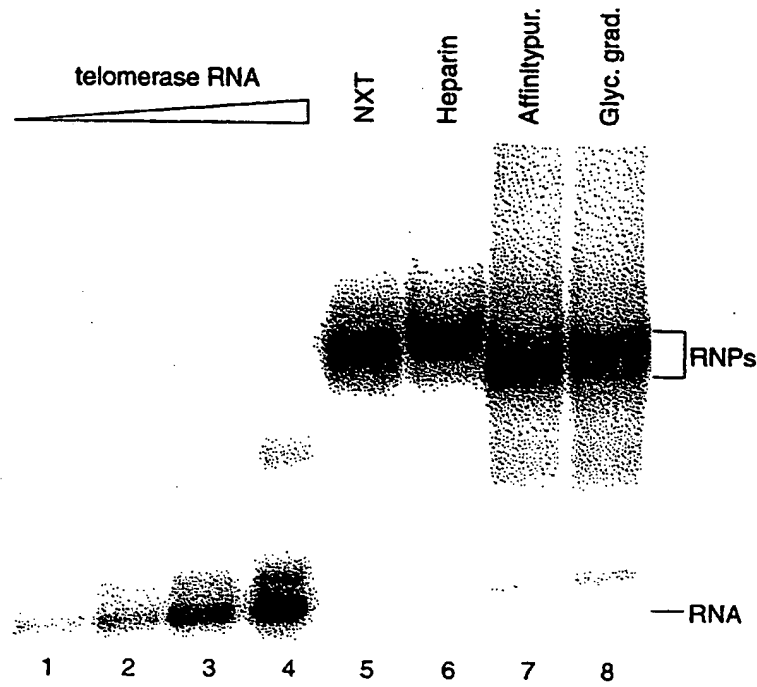


FIG. 27

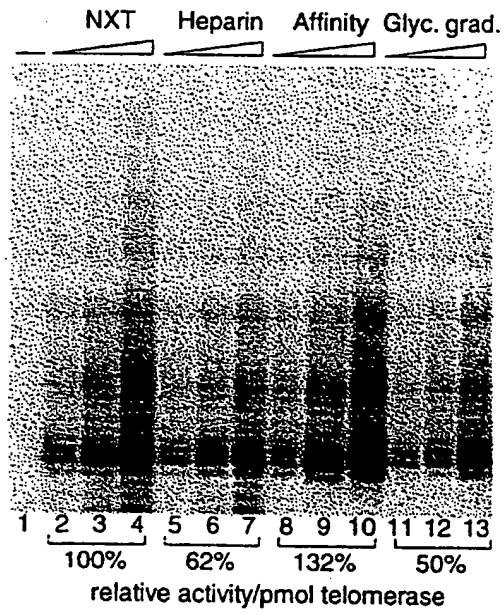


FIG. 28

37/103

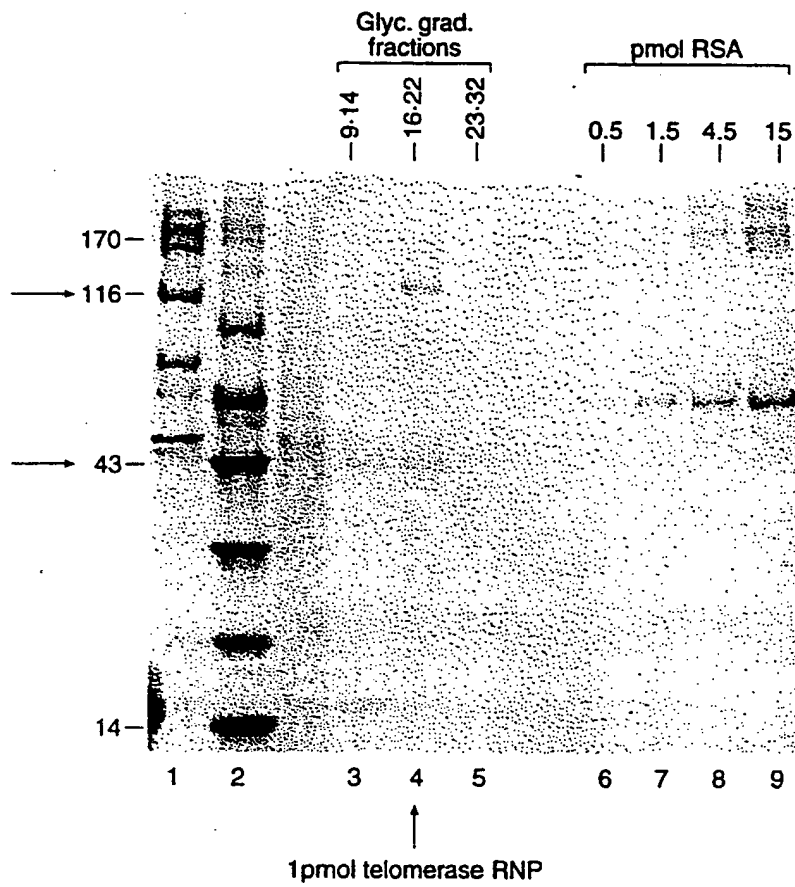


FIG. 29

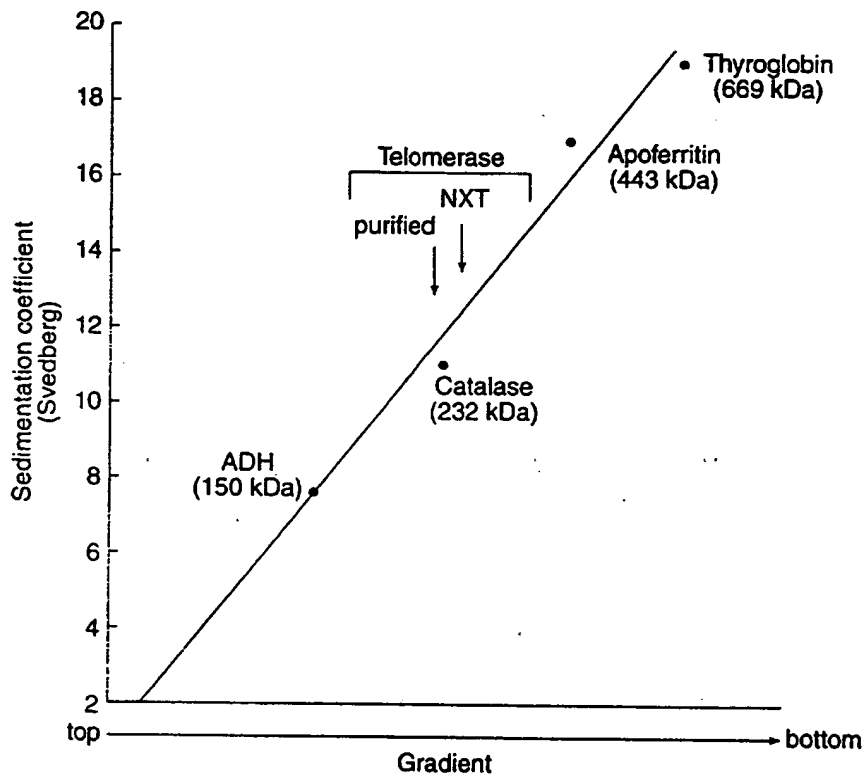


FIG. 30

Telomerase:

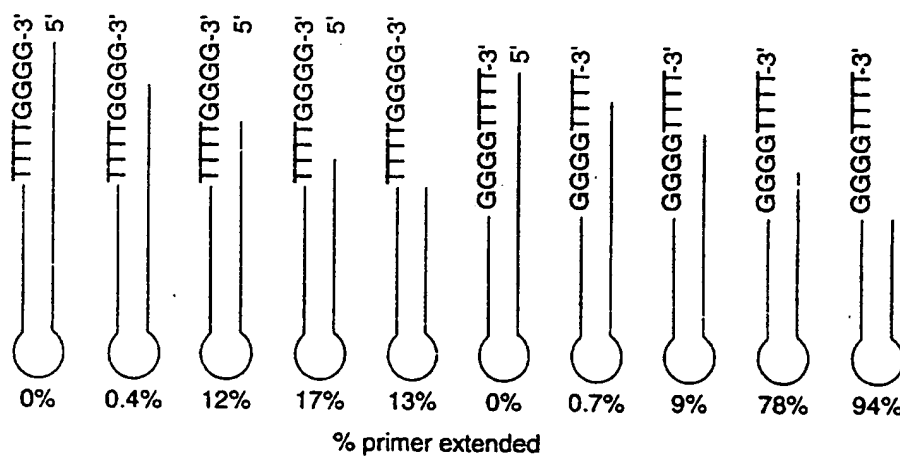
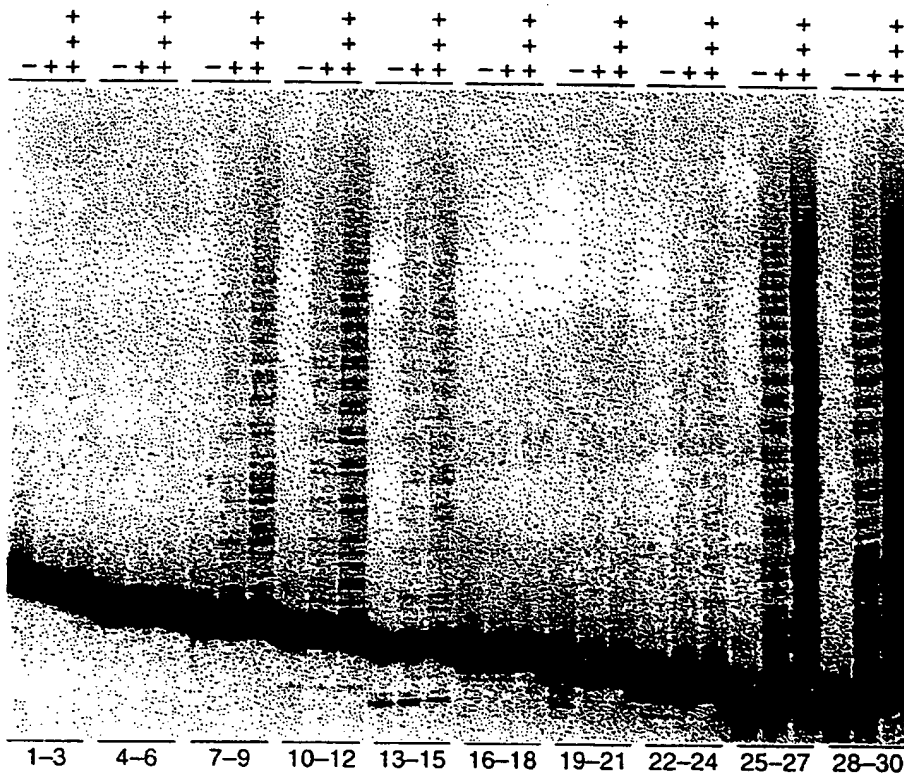


FIG. 31

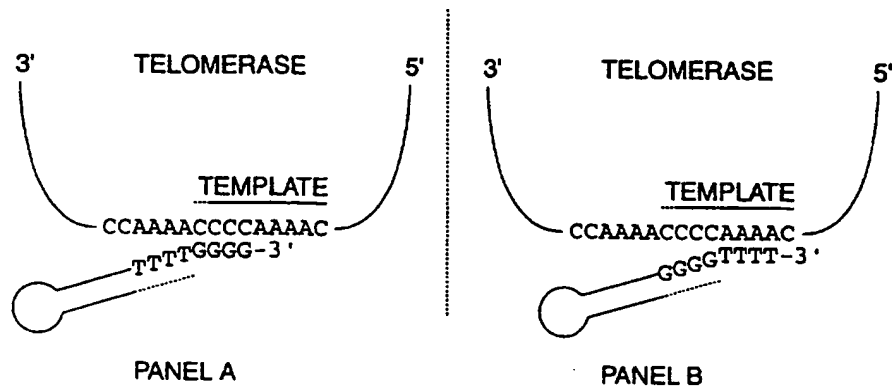


FIG. 32

| | | | | | |
|------|-------------|------------|-------------|-------------|-------------|
| 1 | CCCCAAAACC | CCAAAACCCC | AAAACCCCTA | TAAAAAAGA | AAAAATTGAG |
| 51 | GTAGTTTGA | AATAAAATAT | TATTCCTGCA | CAAAATGGAGA | TGGATATTGA |
| 101 | TTTGGATGAT | ATAGAAAATT | TACTTCCTAA | TACATTCAAC | AAGTATAGCA |
| 151 | GCTCTTGTAG | TGACAAGAAA | GGATGCAAAA | CATTGAAATC | TGGCTCGAAA |
| 201 | TCGCCTTCAT | TGACTATTCC | AAAGTTGCAA | AAACAATTAG | AGTTCTACTT |
| 251 | CTCGGATGCA | AATCTTTATA | ACGATTCTTT | CTTGAGAAAA | TTAGTTTTAA |
| 301 | AAAGCGGAGA | GCAAAGAGTA | GAAATTGAAA | CATTACTAAT | GTTTAAATAA |
| 351 | AATCAGGTAA | TGAGGATTAT | TCTATTTTTT | AGATCACTTC | TTAAGGAGCA |
| 401 | TTATGGAGAA | AATTACTTAA | TACTAAAAGG | TAAACAGTTT | GGATTATTTC |
| 451 | CCTAGCCAAC | AATGATGAGT | ATATTAAATT | CATATGAGAA | TGAGTCAAAG |
| 501 | GATCTCGATA | CATCAGACTT | ACCAAAGACA | AACTCGCTAT | AAAACGCAAG |
| 551 | AAAAAGTTTG | ATAATCGAAC | AGCAGAAGAA | CTTATTGCAT | TTACTATTTCG |
| 601 | TATGGGTTTT | ATTACAATTG | TTTTAGGTAT | CGACGGTGAA | CTCCCGAGTC |
| 651 | TTGAGACAAT | TGAAAAAGCT | GTTTACAAC | GAAGGAATCG | CAGTTCTGAA |
| 701 | AGTTCTGATG | TGTATGCCAT | TATTTTGTTA | ATTAATCTCA | AATATCTTAT |
| 751 | CTCAATTTAA | TGGATAGCTA | TAGAAACAAA | CCAAATAAAC | CATGCAAGTT |
| 801 | TAATGGAATA | TACGTTAAAT | CCTTTGGGAC | AAATGCACAC | TGAATTTATA |
| 851 | TTGGATTCTT | AAAGCATAGA | TACACAGAAT | GCTTTAGAGA | CTGATTTAGC |
| 901 | TTACAACAGA | TTACCTGTTT | TGATTACTCT | TGCTCATCTC | TTATATCTTT |
| 951 | AAAAGAAGCA | GGCGAAATGA | AAAGAAGACT | AAAGAAAAGAG | ATTTCAAAAT |
| 1001 | TTGTTGATTTC | TCTGTAAACC | GGAATTAACA | ACAAGAATAT | TAGCAACGAA |
| 1051 | AAAGAAGAAG | AGCTATCACA | ATCCTGATTC | TTAAAGATTT | CAAAAATTCC |
| 1101 | AGGTAAGAGA | GATACATTCA | TTAAAATTCA | TATATTATAG | TTTTTCATTT |
| 1151 | CACAGCTGTT | ATTTTCTTTT | ATCTTAACAA | TATTTTGTGA | TTAGCTGGAA |
| 1201 | GTAAAAAGTA | TCAAATAAGA | GAAGCGCTAG | ACTGAGGTAA | CTTAGCTTAT |
| 1251 | TCACATTCAT | AGATCGACCT | TCATATATCC | AATACGATGA | TAAGGAAACA |
| 1301 | GCAGTGATCC | GTTTTAAAAA | TAGTGCTATG | AGGACTAAAT | TTTTAGAGTC |
| 1351 | AAGAAATGGA | GCCGAAATCT | TAATCAAAAA | GAATTGCGTC | GATATTGCAA |
| 1401 | AAGAAATCGAA | CTCTAAATCT | TTCTGTTAATA | AGTATTACCA | ATCTTGATTG |
| 1451 | ATTGAAGAGA | TTGACGAGGC | AACTGCACAG | AAGATCATTAA | AAGAAATAAA |
| 1501 | GTAACCTTTTA | TTAATTAGAG | AATAAACTAA | ATTACTAATA | TAGAGATCAG |
| 1551 | CGATCTTCAA | TTGACGAAAT | AAAAGCTGAA | CTAAAGTTAG | ACAATAAAAA |
| 1601 | ATACAAACCT | TGGTCAAAAT | ATTGAGGAAG | GAAAAGAAGA | CCAGTTAGCA |
| 1651 | AAAGAAAAAA | TAAGGCAATA | AATAAAATGA | GTACAGAAGT | GAAGAAATAA |
| 1701 | AAGATTTATT | TTTTTCAATA | ATTTATTGAA | AAGAGGGGTT | TTGGGGTTTT |
| 1751 | GGGGTTTTTG | GG | | | |

FIG. 34

41/103

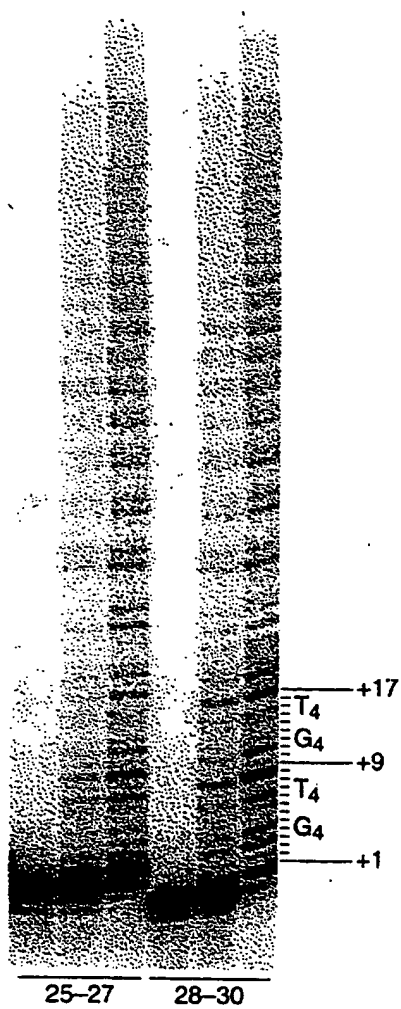


FIG. 33

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
84

FIG. 35

[illegible]

FIG. 35
(CONTINUED)

44/103

901 TTACAACAGATTACCTGTTTTGATTACTCTTGCTCATCTCTTATATCTTTAAAAGAAGCA 960
 AATGTTGTCTAATGGACAAAATAATGAGAACGAGTAGAGAAATATAGAAATTTCTTCGT

a L Q Q I T C F D Y S C S S L I S L K E A -
 b Y N R L P V L I T L A H L L Y L * K K Q -
 c T T D Y L F * L L L L I S Y I F K R S R -

961 GCGGAAATGAAAAGAAGACTAAAGAAAGAGATTTCAAAATTTGTTGATTCTTCTGTAACC 1020
 CCGCTTTACTTTTTCTCTGATTCTTTCTCTAAAGTTTTAAACAATAAGAAGACATTGG

a G E M K R R L K K E I S K F V D S S V T -
 b A K * K E D * R K R F Q N L L I L L * P -
 c R N E K K T K E R D F K I C * F F C N R -

1021 GGAATTAACAACAAGAATATTAGCAACGAAAAAGAAGAGCTATCACAATCCTGATTCT 1080
 CCTTAATTGTTGTTCTTATAATCGTTGCTTTTTCTCTTCGATAGTGTAGGACTAAG

a G I N N K N I S N E K E E E L S Q S * F -
 b E L T T R I L A T K K K K S Y H N P D S -
 c N * Q Q E Y * Q R K R R R A I T I L I L -

1081 TTAAAGATTTCAAAAATTCAGGTAAGAGAGATACATTCAATAAAATTCATATATTATAG 1140
 AATTTCTAAAGTTTTTAAGGTCCATTCTCTCTATGTAAGTAATTTTAAGTATATAATATC

a L K I S K I P G K R D T F I K I H I L * -
 b * R F Q K F Q V R E I H S L K F I Y Y S -
 c K D F K N S R * E R Y I H * N S Y I I V -

1141 TTTTTCATTTACAGCTGTTATTTTCTTTATCTTAACAATATTTTTTGATTAGCTGGAA 1200
 AAAAAGTAAAGTGTGACAAATAAAAGAAAATAGAATTGTTATAAAAACTAATCGACCTT

a F F I S Q L L F S F I L T I F F D * L E -
 b F S F H S C Y F L L S * Q Y F L I S W K -
 c F H F T A V I F F Y L N N I F * L A G S -

1201 GTAAAAAGTATCAAATAAGAGAAGCGCTAGACTGAGGTAAGCTTAGCTTATTCACATTCAT 1260
 CATTTTTCATAGTTTATTCTCTTCGCGATCTGACTCCATTGAATCGAATAAGTGAAGTA

a V K S I K * E K R * T E V T * L I H I H -
 b * K V S N K R S A R L R * L S L F T F I -
 c K K Y Q I R E A L D * G N L A Y S H S * -

1261 AGATCGACCTTCATATATCCAATACGATGATAAGGAAACAGCAGTCATCCGTTTTAAAAA 1320
 TCTAGCTGGAAGTATATAGGTTATGCTACTATTCTTTGTCGTCAGTAGGCAAAATTTTT

a R S T F I Y P I R * * G N S S H P F * K -
 b D R P S Y I Q Y D D K E T A V I R F K N -
 c I D L H I S N T M I R K Q Q S S V L K I -

1321 TAGTGCTATGAGGACTAAATTTTATAGAGTCAAGAAATGGAGCCGAAATCTTAATCAAAAA 1380
 ATCAGGATACTCTGATTTAAAAATCTCAGTTCTTTACCTCGGCTTAGAATTAGTTTTT

a * C Y E D * I F R V K K W S R N L N Q K -
 b S A M R T K F L E S R N G A E I L I K K -
 c V L * G L N F * S Q E M E P K S * S K R -

FIG. 35
 (CONTINUED)

1381 GAATTGCGTCGATATTGCAAAAGAATCGAACTCTAAATCTTTTCGTTAATAAGTATTACCA
 -----+-----+-----+-----+-----+-----+-----+-----+ 1440
 CTTAACGCAGCTATAACGTTTCTTAGCTTGAGATTTAGAAAGCAATTATTTCATAATGGT

 a E L R R Y C K R I E L * I F R * * V L P -
 b N C V D I A K E S N S K S F V N K Y Y Q -
 c I A S I L Q K N R T L N L S L I S I T N -

 ATCTTGATTGATTGAAGAGATTGACGAGGCAACTGCACAGAAGATCATTAAAGAAATAAA
 1441 -----+-----+-----+-----+-----+-----+-----+ 1500
 TAGAACTAACTAACTTCTCTAACTGCTCCGTTGACGTGTCTTCTAGTAATTTCTTTATTT

 a I L I D C R D * R G N C T E D H * R N K -
 b S * L I E E I D E A T A Q K I I K E I K -
 c L D * L K R L T R Q L H R R S L K K * S -

 GTAACCTTTTATTAATTAGAGAATAAACTAAATTACTAATATAGAGATCAGCGATCTTCAA
 1501 -----+-----+-----+-----+-----+-----+-----+ 1560
 CATTGAAAATAATTAATCTCTTATTGATTTAATGATTATATCTCTAGTCGCTAGAAGTT

 a V T F I N * R I N * I T N I E I S D L Q -
 b * L L L I R E * T K L L I * R S A I F N -
 c N F Y * L E N K L N Y * Y R D Q R S S I -

 TTGACGAAATAAAAGCTGAACTAAAGTTAGACAATAAAAAATACAAACCTTGGTCAAAAT
 1561 -----+-----+-----+-----+-----+-----+-----+ 1620
 AACTGCTTTATTTTCGACTTGATTTCATCTGTTATTTTTATGTTTGAACCAAGTTTAA

 a L T K * K L N * S * T I K N T N L G Q N -
 b * R N K S * T K V R Q * K I Q T L V K I -
 c D E I K A E L K L D N K K Y K P W S K Y -

 ATTGAGGAAGGAAAAGAAGACCAGTTAGCAAAAGAAAAATAAGGCAATAAATAAAATGA
 1621 -----+-----+-----+-----+-----+-----+-----+ 1680
 TAACCTCCTTCTTTCTTCTGGTCAATCGTTTCTTTTATTCGTTATTTATTTTACT

 a I E E G K E D Q L A K E K I R Q * I K * -
 b L R K E K K T S * Q K K K * G N K * N E -
 c * G R K R R P V S K R K N K A I N K M S -

 GTACAGAAGTGAAGAAATAAAAGATTTATTTTTTTCAATAATTTATTGAAAAGAGGGGTT
 1681 -----+-----+-----+-----+-----+-----+-----+ 1740
 CATGCTTCACTTCTTTATTTTCTAAATAAAAAAGTTATTAATAAATTTCTCCCCAA

 a V Q K * R N K R F I F F N N L L K R G V -
 b Y R S E E I K D L F F S I I Y * K E G F -
 c T E V K K * K I Y F F Q * F I E K R G F -

 TTGGGGTTTTGGGGTTTTGGGG
 1741 -----+-----+-----+-----+-----+-----+-----+ 1762
 AACCCCAAAACCCCAAAACCC

 a L G F W G F G -
 b W G F G V L G -
 c G V L G F W -

FIG. 35
(CONTINUED)

— — — — —

FIG. 36

47/103

798 EKLINVSRENGFKFNMKK.LQTSFPLSPSKFAKYGMDSVEEQNIVQDYCD 846
 577PGDELRPSPQKLLQEKGLGGG..TDFPYECIDEWTKNKTHTVD 617
 847 WIGISIDMKTLALMPNINLRIGILCTLNLMQTKKASMWLKKKLSFLM 896
 618 NIVILSDMMIAEGYSDINVRGSSIVNSI.....KKYKDEVN 653
 897 NNITHYFRKTITTEDFANKTLNKLFISSGGYKYMCAKEYKD.HFKKNLAM 945
 654 PNIKIF...AVDLEGYG.....KCLNLGDEFNENNYIKIFGM 687
 946 SSMIDLEVSKIIYSVTRAFFKYLVCNIKDTIFGEEHYPDFFLSTLKHFI 995
 688 SDSI.....LKFISAKQGA.....NMVE 706
 996 IFSTKKYIFNRVC 1008
 707 VI..KNFALQKIG 717

FIG. 36
(CONTINUED)

132 LSTQKQYFFQDEWNQVRAMIGNEL.FRHLYTKYLIFORTSE..GTLVQFC 178
 1 MSRRNQ.....KKPQAPIGNETNLDFVLQNLVYKSQIEHYKTQQQQI 43
 179 GNNVFDHLKVNDKFDKKQKGAADMNEPRCCSTCKYNVKNKDHFLNNIN 228
 44 KEEDLKLKFKNQDQDGNNGDDDEE.....NNSNKQQLLRRVN 84
 229 VPNNWNMKSRTTRIFYCTHFNRRNQFFKKHEFVSNKNNISAMDRAQTIFTN 278
 85QIKQQVQLIKK...VGSKEVDLNLNEDENKKN 114
 279 IFRFNIRKRLKDKVIEKIAIYMLEKVDFNFNYLTKSCPLPENWRERKQ 328
 115 GLSEQQVKEEQLRTITEEQVKYQNLVFNMDYQLDLNESGGHRRHRET DY 164
 329 KIENLINKTREESKYEEFSTYTTDNKCVTQFINE.FFYNILPKDFLTG 377
 165 DTEKWFEISHDQK.....NYVSIYANQKTSYCWKLKDYFNK 200
 378 RNRKNFQKKVKYVELNKHLEIHNLLLEKINTREISWMQVETSAKHFY 427
 201 NNYDHLNVSINRLE..TEAEFYAFDDFSQTIKLTNNSYQTVNID..... 242
 428 FDHENIYVLWKLLRWI..FEDLVVSLIRCFYVTEQQKSYSKTYYYRKNI 475
 243 VNFDNNLCILALLRFLSLERFNILNIRSSY..TRNQYNFEKIGELLETI 290
 476 WDVIMKMSIADLKKETLAEVQKEVEEWKSLGFAPGKLRLIPKTTFRP 525
 291 FAVVFSHR.....HLQGIHLQVPCEAFQYLVNSSSQISVKDSQLQ 330
 526 IMTFNKKIVNSDRKTTKLTNTKLLNSHMLKTLKNRMFKDPFGFAVFNY 575
 331 VYSFSTDLKLVD..TNKVQDYFKFLQEFPRLTHVSQQAIPVSATNAVENL 378

FIG. 37

49/103

```

1 MEMDIDLDIENL.....LPNTFNKYSSSCSDKKGCKTLKSGSKSPS... 42
  ||:|.:.|||...||:|.:.|||...||:|.:.|||...||:|.:.|||...
491 IELAIAVKNLDEIKGHTAIFSDVSGSMSTSMGGAKKYGSVRTCLEC 540

43 .LTIPKLQKQ.....LEFYFSDANLYNDSFLRKLVLKSQEQRVEIETLL 85
  ||:|.:.|||...||:|.:.|||...||:|.:.|||...||:|.:.|||...
541 ALVLGLMVKQRCEKSSFYIFSSPSSQCNKCYL.EVDLPGDEL RPSMQKLL 589
  
```

FIG. 39

```

telomerase p43 LQKLEFYFSDANLYNDSFLRKLVLKSQEQRVEIETLLM
human La ICHQVEYFYGDFNLPRDKFLKEQI.KLDEGWVPLEIMIK
Xenopus LaA ICEQIEYFYGDFNLPRDKFLKQOI.LLDDGWVPLETMIK
Drosophila La ILROVEYFYGDFNLNRDKFLREQIGKNEDGWVPLSVLVT
S. c. Lhplp CLKQVEFYFSEFNFPYDRFLRTTAEK.NDGWVPISTIAT
  
```

FIG. 41

```

1 aactcattta attactaatt taatcaacaa gattgataaa aagcagtaaa taaaacccaa
61 tagatttaatt ttgaaagta tcaattgaaa aatggaaatt gaaaacaact aagcacaata
121 gccaaaagcc gaaaaattgt ggtgggaact tgaattagag atgcaagaaa accaaaatga
181 tatataagtt aggggttaaga ttgacgatcc taagcaatat ctggtgaacg tcaactgcagc
241 atgtttgttg taggaaggtg gttactacta agataaagat gaaagaagat atatcatcac
301 taaagcactt cttgaggttg ctgagttctga tcctgagttc atctgctagt tggcagttct
361 catccgtaat gaactttaca tcagaactac cactaactac attgtagcat tttgtgttgt
421 ccacaagaat actcaaccat tcctcgaaaa gtacttcaac aaagcagtac ttttgcctaa
481 tgacttactg gaagtctgtg aatttgcata gggtctctat atttttgatg caactgaatt
541 caaaaatttg tatcttgata ggatactttc ataagatatt cgtaaggaac tcactttccg
601 taagtgttta caaagatgcy tcagaagcaa gttttctgaa ttcaacgaat actaacttgg
661 taagtattgc actgaatcct aacgtaagaa aacaatgttc cgttacctct cagttaccaa
721 caagtaaaag tgggattaaa ctaagaagaa gagaaaagag aatctcttaa ccaaacttta
781 ggcaataaag gaactctgaag ataagtccaa gagagaaact ggagacataa tgaacgttga
841 agatgcaatc aaggctttta aaccagcagt tatgaagaaa atagccaaga gatagaattg
901 catgaagaaa cacatgaagg cacctaaaat tcctaactct accttggaat caaagtactt
961 gaccttcaag gatctcatta agttctgcca tatttctgag cctaaagaaa gagtctataa
1021 gatccttggt aaaaaatacc ctaagaccga agaggaatac aaagcagcct ttggtgattc
1081 tgcatctgca cccttcaatc ctgaattggc tggaaagcgt atgaagattg aaatctctaa
1141 aacatgggaa aatgaactca gtgcaaaagg caacactgct gaggtttggg ataatttaat
1201 ttcaagcaat taactcccat atatggccat gttacgtaac ttgtctaaca tcttaaaagc
1261 cggtgtttca gatactacac actctattgt gatcaacaag atttgtgagc ccaaggccgt
1321 tgagaactcc aagatgttcc ctcttcaatt ctttagtgcc attgaagctg ttaatgaagc
1381 agttactaag ggattcaagg ccaagaagag agaaaatatg aatcttaag gtcaaatcga
1441 agcagtaaaag gaagttgttg aaaaaaccga tgaagagaag aaagatatgg agttggagta
1501 aaccgaagaa ggagaatttg ttaaagtcaa cgaagggaatt ggcaagcaat acattaactc
1561 cattgaactt gcaatcaaga tagcagttaa caagaattta gatgaaatca aaggacacac
1621 tgcaatcttc tctgatgttt ctggttctat gagtacctca atgtcaggtg gagccaagaa
1681 gtatgggttc gttcgtactt gttcgtgagtg tgcattagtc cttggtttga tggtaaaata
1741 acgttgtgaa aagtctctcat tctacatctt cagttcacct agttctcaat gcaataagtg
1801 ttacttagaa gttgatctcc ctggagacga actccgtcct tctatgtaaa aacttttgca
1861 agagaaagga aaacttggtg gtggtactga tttcccctat gagtgcattg atgaatggac
1921 aaagaataaa actcacgtag acaatatcgt tattttgtct gatatgatga ttgcagaagg
1981 atattcagat atcaatgtta gaggcagttc cattgttaac agcatcaaaa agtacaagga
2041 tgaagtaaat cctaacatta aaatctttgc agttgactta gaaggttacg gaaagtgcct
2101 taatctagggt gatgagttca atgaaaacaa ctacatcaag atattcggta tgagcgattc
2161 aatcttaaaag ttcatttcag ccaagcaagg agggagcaaat atgggtcgaag ttatcaaaaa
2221 ctttgccctt caaaaaatag gacaaaagtg agtttcttga gattcttcta taacaaaaat
2281 ctcacccac ttttttgttt tattgcatag ccattatgaa atttaaatta ttatctattt
2341 atttaagtta cttacatagt ttatgtatcg cagtcattata gcctattcaa atgattctgc
2401 aaagaacaaa aaagattaaa a
  
```

FIG. 42

| | Motif A | Motif B |
|--------------------|---|--------------|
| Consensus | h--hDh---h--h | h---+QD---gp |
| telomerase p123 | QPKLFATMDIEKCYDSVNRKLSFLKTTKLL-100-KFYKQTKGIQGLCVSSLSFFYATLEESSLGFL | |
| Dong (LINE) | KRNHLCTYDDYKKAFOISPHSWLIQVLEIYKIN-28-RQIAIKKGIYQDSDLSPLWFCALNPLSHQLHNR | |
| al S.c. (group II) | FGGSNWFREVDLKKCFDTISHDLIIKELKRYISD-26-HVPVGPVRCVQCAPTSALCNNAVLLRDLRRLAGLA | |
| HIV-RT | LKKKSVTVLVDVGDAYFSVPLDEDFRKYTAFTIP-7-GIRYQYNVLPQGWKGSFAIFQSSMTKILEPPFKQN | |
| L8543.12 | VLPELYFMKFDVKSCYDSIPRMECMRILKDALKN-68-KCYIREDEGLFQSSLSAPIVDLVYDDLLEFYSEFK | |
| | Motif C | Motif D |
| Consensus | h--YhDdhhh | Gh-h---K |
| telomerase p123 | -14-LMRLTDDYLLITTTQENN-0-AVLFIKLIIVSRENGKFNMKKLOT-23-QDYCDWIGISI | |
| Dong (LINE) | -16-HLIYMDIDIKLYAKNDKE-0-MKKLIDTTTTSNDISWQGLDKCT-25-KCLYKYLQFQQ | |
| al S.c. (group II) | -55-YVRYADDILIGVLGSKN-2-KTIKRDNLNPLNS.LGLTINEEKTLL-4-ETPARFLGYNI | |
| HIV-RT | -4-IYQYMDLGVGSHLEIG-1-HRTKIEELRQHLRWGLTTPDKKHQK-0-EPPFLMWGYEL | |
| L8543.12 | -8-ILKLADDFLIISTDQQQ.....VINIKKLMGFGQYNKAKNR-41-IRSKSKGIFR | |
| | Motif E | |
| | | h-hLgh-h |

FIG. 40

51/013

MEIENNQAQQPKAEKLWWELELEMQENQNDIQVRVKIDDPKQYL
VNVTAACLLQEGSYQDKDERRYIITKALLEVAESDPEFICQLA
VYIRNELYIRTTTNYIVAFVVKNTQPFIEKYFNKAVLLPNDL
LEVCEFAQVLYIFDATEFKNLYLDRILSQDIRKELTFRKCLQRC
VRSKFSEFNEYQLGKYCTESQRKKTMFRLSVTNKQKWDQTKKK
RKENLLTKLQAIKESEDKSKRETGDIMNVEDAIKALKPAVMKKI
AKRQNAKKHMKAPKIPNSTLESKYLTFFKDLIKFCHISEPKERV
YKILGKKYPKTEEEYKAAFSDSASAPFNPPELAGKRMKIEISKTW
ENELSAKGNTAEVWDNLISSNQLPYMAMLRNLSNLIKAGVSDTT
HSIVINKICEPKAVENSKMFPLOFFSAIEAVNEAVTKGFKAKKR
ENMNLKGQIEAVKEVVEKTDEEKKDMELEQTEEGEFVKVNEGIG
KQYINSIELAIAVKNLDEIKGHTAIFSDVSGSMSTSMSSGA
KKYGSVRTCLECALVLGLMVQRCESFFYIFSSPSSQCNKCYL
EVDLPGLDELPSMQKLLQEKGLGGGTDFPYECIDEWTKNKTTHV
DNIVILSDMMIAEGYSDINVRGSSIVNSIKKYKDEVNPNKIFA
VDLEGYKCLNLGDEFNENNYIKIFGMSDSILKFISAKQGGANM
VEVIKNFALQKIGQK

FIG. 43

MSRRNQKKPQAPIGNETNLDFVLQNLLEVYKSQIEHYKTQQQIK
EEDLKLKFKNQDQDGNNGNDDEENNSNKQELLRRVNQIKQ
QVQLIKKVGSKVEKDLNLNEDENKKNGLSEQQVKEEQLRITEE
QVKYQNLVFNMDYQDLNLSGGHRRHRRETQDYTEKWFESHDQ
KNYVSIYANQTSYCWWLKDYFNKNYDHLNVSINRLETEAEFY
AFDDFSQTIKLTNNSYQTVNIDVNFNNLCILALLRFLSLERF
NILNIRSSYTRNQYNFEKIGELLETFVAVFSHRHLOGIHLQVP
CEAFQYLVNSSSQISVKSQQLQVYSFSTDLKLVDTNKVQDYFKF
LQEFPRLTHVSQQAIPVSATNAVENLNVLLKKVKHANLNVSI
TQFNDFDYFVNLQHLKLEFGLPNILTKQKLENLLLSIKQSKNL
KFLRLNFYTYVAQETSRKQILKQATTIKNLKNKNQEEETPETKD
ETPSESTSGMKFFDHLSELTELEDFSVNLQATQEIYDSLHLLI
RSTNLKKFKLSYKYEMEKSKMDTFIDLKNIYETLNNLKRCVNI
SNPHGNISYELTNKDSTFYKFKLTNLQELQHAKYTFKQNEFQFN
NVKSAKIESSSLESLEDIDSLCKSIASCKNLQNVNI IASLLYPN
NIQKNPFPKNPPLLFFKQFEQLKNLENVINCILDQHILNSISEF
LEKNKKIKAFILKRYLLQYLLDYTKLFTLQQLPELNQVYINQ
QLEELTVSEVHKQVWENHKQAFYEPLCEFIKESSTQLQIDFD
QNTVSDDSIKKILESISESKYHHYLRNPSQSSSLIKSENEEIQ
ELLKACDEKGLVKAYYKFPCLCLPTGTYDYNSDRW

FIG. 45

MKILFEFIQDKLDIDLQTNSTYKENLKGHFNGLDEILTTCFAL
PNSRKIALPCLPGDLSHKAVIDHCIIYLLTGELYNNVLTFGYKI
ARNEDVNNSLFCHSANVNVTLKGAAWKMFHSLVGTAFVLLI
NYTVIQFNGQFFTQIVGNRCNEPPLPKWVORSSSSSATAAQIK
QLTEPVTNKQFLHKLINSSSFFPYSKILPSSSSIKKLTDLREA
IFPTNLVKIPQRLKVRINLTQKLLKRHKRLNYVSIILNSICPPL
EGTVLDLSHLSRQSPKERVLFIIIVILQKLLPQEMFGSKKNKGK
I IKNLNLLSLPLNGYLPFDSLLKRLKDFRWLFISDIWFTKH
NFENLNQLAICFISWLFRLIPKIIQTFYCTEISSTVTIVYFR
HDTWNKLTTPFIVEYFKTYLVENNVCRNHSYTLNPNHSMKRI
IPKKSNNEFRIIAIPCRGADEEEFTIYKENHKNAIQPTQKILEY
LRNKRPTSFTKIYSPTQIADRIKEFKQRLKKNVNLPELYFMK
FDVKSCYDSIPRMECMRILKDALKNENGFFVRSQYFFNTNTGV
KLFNVVNASRVKPYELYIDNVRTVHLSNQDVINVVEMEIFKTA
LWVEDKCYIREDGLFGSSLSAPIVDLVYDDLLEFYSEFKASPS
QDTLILKLADDFLIISTDQQVINIKKLAMGGFQKYNANRDK
ILAVSSQSDDDTVIQFCAMHIFVKELEVWKHSSTMMNFHRSKS
SKGIFRSLIALFNTRISYKTIDTNLSTNTVLMQIDHVVKNISE
CYKSAFKDLSINVTQNMQFHSFLQRIEMTVSGCPITKCDPLIE
YEVFTILNGFLESLSNTSKFDNIILLRKEIQHLQAYIYIYI
HIVN

FIG. 46

```

1 tcaatactat taattaataa ataaaaaaa gcaaaactaca aagaaaaatgt caaggcgtaa
61 ctaaaaaaag ccataggctc ctataggcaa tgaaacaaat cttgattttg tattacaaaa
121 tctagaagtt tacaaaagcc agattgagca ttataagacc tagtagtaat agatcaaaga
181 ggaggatctc aagcttttaa agttcaaaaa ttaagattag gatggaaact ctggcaacga
241 tgatgatgat gaagaaaaa actcaaataa ataataagaa ttattaagga gagtcaatta
301 gattaagtag caagtttaat tgataaaaa agttgggttct aaggtagaga aagatttgaa
361 tttgaacgaa gatgaaaaca aaaagaatgg actttctgaa tagcaagtga aagaagagta
421 attaagaacg attactgaag aatagggttaa gtattaaaat ttagtattta acatggacta
481 ccagtttagat ttaaattgaga gtgggtggcca tagaagacac agaagagaaa cagattatga
541 tactgaaaaa tgggtttgaaa tatctcatga ccaaaaaaat tatgtatcaa tttacgccaa
601 ctaaaagaca tcatattggtt ggtgggttaa agattatttt aataaaaaa attatgatca
661 tcttaatgta agcattaaca gactagaaac tgaagccgaa ttctatgcct ttgatgattt
721 ttcacaaaca atcaaaactta ctaataattc ttactagact gttaacatag acgttaattt
781 tgataataat ctctgtatac tcgcattgct tagattttta ttatcactag aaagattcaa
841 tattttgaat ataagatctt cttatacaag aaattaatat aattttgaga aaattgggtga
901 gctacttgaa actatcttctg cagttgttctt ttctcatgc cacttacaag gcattcattt
961 acaagttcct tgcgaaagcg tctaataattt agttaactcc tcatcataaa tttagcgtaa
1021 agatagctaa ttatagggtat actctttctc tacagactta aaattagttg acactaacia
1081 agtccaagat tattttaagt tcttataaga attcctcgt ttgactcatg taagctagta
1141 ggctatccca gtttagtgcta ctaacgctgt agagaacctc aatgttttac ttaaaaaggt
1201 caagcatgct aatcttaatt tagtttctat ccctacctaa ttcaattttg atttctactt
1261 tgttaattta taacatttga aattagagtt tggattagaa ccaaatattt tgacaaaaa
1321 aaagcttgaa aatctacttt tgagtataaa ataatacaaaa aatcttaaat ttttaagatt
1381 aaacttttac acctacgttg cttagaagaa cctcagaaaa cagatattaa aacaagctac
1441 aacaatcaaa aatctcaaaa acaataaaaa tcaagaagaa actcctgaaa ctaagatga
1501 aactccaagc gaaagcacaa gtgggtatgaa attttttgat catcttctg aattaaccga
1561 gcttgaagat ttcagcgtaa acttgtaagc taccgaagaa atttatgata gcttgacaaa
1621 acttttgatt agatcaacaa atttaagaa gttcaaatta agttacaaat atgaaatgga
1681 aaagagtaaa atggatacat tcatagatct taagaatatt tatgaaacct taaacaatct
1741 taaaagatgc tctgttaata tatcaaatcc tcatggaaac atttcttatg aactgacaaa
1801 taaagattct actttttata aatttaagct gaccttaaac taagaattat aacacgctaa
1861 gtatactttt aagtagaacg aattttaatt taataacggt aaaagtgcaa aaattgaatc
1921 ttcttcatta gaaagcttag aagatattga tagtctttgc aaatctattg cttcttgtaa
1981 aaatttacaa aatgttaata ttatcgccag ttgtctctat cccaacaata tttagaaaaa
2041 tcctttcaat aagcccaatc ttctattttt caagcaattt gaataattga aaaatttgga
2101 aaatgtatct atcaactgta ttcttgatca gcatatactt aattctattt cagaattctt
2161 agaaaagaat aaaaaaataa aagcattcat tttgaaaaga tattatttat tacaatatta
2221 tcttgattat actaaattat ttaaaacact tcaatagtta cctgaattaa attagttta
2281 cattaattag caattagaag aattgactgt gagtgaagta cataagtaag tatgggaaaa
2341 ccacaagcaa aaagctttct atgaaccatt atgtgagttt atcaaagaat catcctaaac
2401 ccttttagcta atagattttg accaaaacac tgtaagtgat gactctatta aaaagatttt
2461 agaattctata tctgagtcta agtatcatca ttatttgaga ttgaacccta gttaatctag
2521 cagtttaatt aaatctgaaa acgaagaaat ttaagaactt ctcaaagctt gcgacgaaaa
2581 aggtgtttta gtaaaagcat actataaatt ccctctatgt ttaccaactg gtacttatta
2641 cgattacaat tcagatagat ggtgattaat taaatattag tttaaataaa tattaaatat
2701 tgaatatttc tttgcttatt atttgaataa tacatacaat agtcattttt agtggttttga
2761 atatatttta gttatttaat tcattatttt aagtaataaa ttatttttca atcatttttt
2821 aaaaaatcg

```

FIG. 44

Oxytricha
Euplotes

LCVSYILSSFYYANLEENALQFLRKESMDPEKPETNLLMRLT
LCVSSILSSFYYATLEESSIGFLRDESMNPENPNVNLMLRLT

FIG. 47

ATTTATACTCATGAAAATCTTATTTCGAGTTCATTCAAGACAAGCTTGACATTGATCTACA
GACCAACAGTACTTACAAAGAAAAATTTAAAATGTGGTCACTTCAATGGCCTCGATGAAAT
TCTAACTACGTGTTTCGCAC TACCAAATTCAGAAAAATAGCATTACCATGCCTTCCTGG
TGACTTAAGCCACAAAGCAGTCATTGATCACTGCATCATTACCTGTTGACGGGCGAATT
ATACAACAACGTACTAACAATTTGGCTATAAAAATAGCTAGAAATGAAGATGTCAACAATAG
TCTTTTTTGCCATTCTGCAAAATGTTAACGTTACGTTACTGAAAGGCGCTGCTTGAAAAAT
GTTCCACAGTTTGGTTCGGTACATACGCATTTCGTTGATTTATTGATCAATTATACAGTAAT
TCAATTTAATGGGCAGTTTTTCACTCAAATCGTGGGTAACAGATGTAACGAACCTCATCT
GCCGCCCAAATGGGTCCAACGATCATCTCATCCGCAACTGCTGCGCAAATCAAACA
ACTTACAGAACCAGTGACAAATAAACCAATTCTTACACAAGCTCAATATAAAATTCCTCTTC
TTTTTTTCTTATAGCAAGATCCTTCCTTCATCATCATCTATCAAAAAGCTAACTGACTT
GAGAGAAGCTATTTTTTCCCACAAATTTGGTTAAAAATTCCTCAGAGACTAAAGGTACGAAT
TAATTTGACGCTGCAAAAGCTATTAAAGAGACATAAGCGTTTGAATTACGTTTCTATTTTT
GAATAGTATTTTGCCCAACCATTTGGAAGGGACCGTATTGGACTTGTGCGATTGAGTAGGCA
ATCACCAAAGGAACGAGTCTTGAAATTTATCATTTGTTATTTTACAGAAGTTATTACCCCA
AGAAATGTTTGGCTCAAAGAAAAATAAGGAAAAATTTATCAAGAATCTAAATCTTTTATT
AAGTTTACCCTTAAATGGCTATTTACCATTGTAGTTTGTGAAAAAGTTAAGATTAAA
GGATTTTCGGTGGTTGTTCAATTTCTGATATTGGTTTACCAAGCACAAATTTGAAAACTT
GAATCAATTGGCGATTTGTTTCAATTTCTGGCTATTAGACAATAATTTCCCAAAATTAT
ACAGACTTTTTTTTACTGCAACCGAAATATCTTCTACAGTGACAATTGTTTACTTTAGACA
TGATACTTGAATAAACTTATCACCCCTTTTATCGTAGAATATTTTAAAGACGTAAGT
CGAAAAACAACGTATGTAGAAACCATAATAGTTACACGTTGTCCAATTTCAATCATAGCAA
AATGAGGATTATACCAAAAAAAGTAATAATGAGTTTCAAGGATTATGGCATCCCATGACG
AGGGGCAGACGAAGAAGAAATTCACAATTTATAAGGAGAATCACAAAAATGCTATCCAGCC
CACTCAAAAAATTTTAGAATACCTAAGAAACAAAAGGCCGACTAGTTTACTAAAAATATA
TTCTCCAACGCAATAGCTGACCGTATCAAAGAAATTTAAGCAGAGACTTTTAAAGAAATT
TAATAATGTCTTACCAGAGCTTTATTTTCAATGAAATTTGATGTCAAATCTTCTATGATTC
CATACCAAGGATGGAATGTATGAGGATACTCAAGGATGCGCTAAAAATGAAATGGGTT
TTTCGTTAGATCTCAATATTTCTTCAATACCAATACAGGTGTATTGAAGTTATTTAATGT
TGTTAACGCTAGCAGAGTACCAAAACCTTATGAGCTATACATAGATAATGTGAGGACGGT
TCATTTATCAAATCAGGATGTTATAAACGTTGTAGAGATGGAATATTTAAACAGCTTT
GTGGGTTGAAGATAAGTGCTACATTAGAGAAGATGGTCTTTTTTCAGGGCTCTAGTTTATC
TGCTCCGATCGTTGATTTGGTGTATGACGATCTTCTGGAGTTTATAGCGAGTTTAAAGC
CAGTCTTAGCCAGGACACATTAATTTTAAACTGGCTGACGATTTCTTATAATATCAAC
AGACCAACAGCAAGTATCAATATCAAAAAGCTTGCCATGGGCGGATTTCAAAAATATAA
TGCGAAAGCCAATAGAGACAAAATTTTAGCCGTAAGCTCCCAATCAGATGATGATACGGT
TATTCATTTTGTGCAATGCACATTTGTTAAAGAATTGGAAGTTTGGAAACATTTCAAG
CACAATGAATAATTTCCATATCCGTTTCGAAATCTAGTAAAGGGATATTTTCAAGTTTAAAT
AGCGCTGTTTAACTAGAAATCTCTTATAAAACAATTGACACAAATTTAAATTCAACAAA
CACCGTTCTCATGCAAAATGATCATGTTGTAAAGAACATTTTCGGAATGTTATAAATCTGC
TTTTAAGGATCTATCAATTAATGTTACGCAAAATATGCAATTTTCATTCGTTCTTACAACG
CATATTGAAATGACAGTCAGCGTTGTCCAATTACGAAATGTGATCCTTTAATCGAGTA
TGAGGTACGATTACCATATTGAATGGATTTTGGAAAGCCTATCTTCAAAACACATCAAA
ATTTAAAGATAATATCATTCTTTTGAGAAAGGAAATTCACACTTGCAAGC

FIG. 48

FIG. 50

— — — — —

FIG. 51

MTEHHTPKSRILRFLNQYVYLCTLNDYVQLVLRGSPASSYSNICERLRSVDQTSFSIFLHSTVVG
 DSKPDEGVQFSSPKCSQSELIANVVKQMFDESERRRNLMLKGFSMNHEDFRAMHNGVQNDLVSTF
 PNYLISILESKNWQLLEIIGSDAMHYLLSKGSIFEALPNDNYLQISGIPLFKNNVFEETVSKKRKR
 TIETSIQNK SARKEVSWNSISISRFSIFYRSSYKKFKQDLVFNLHSHICDRNTVHMWLQWIFPRQFG
 LINAQVQVKQLHKVIPLVQSQTVPVKRLLVYPLIEQTAKRLHRISLSKVYNHYCPYIDTHDEKILS
 YSLKPNQVAFRLSILVRVFPKLIWGNQRIFEIILKDLETFLKLSRYESSFLHYLMSNIKISEIEWL
 VLGRSNNAKMLCLSDPEKRRQIFAEFIYWLNSFIIPILQTSFFYITESTDLRNRVTYFVRKDIWKLLCR
 PFITSMKMEAF EKINENNVRMDTQKTTLP PAVIRLLPKKNTFR LITNLRKFLIKMGSNKMLVSTN
 QTLRPVASILKHLIN EESSGIPFNLEVYMKLLTFKKDLKHRMFG RKKYFVRIDIKSCYDRIKQDLM
 FRIVKKKLKDP EFVIRKYATI HATSDRATKNFVSEAFSYFDMVPFEKVQLLSMKTSDTL FVDFVDY
 WTKSSSEIFKMLKEHLSGHIVKIGNSQYLQKVGIPQGSILSSFLCHFYMEDLIDEYLSFTKKKGSVL
 LRVVDRSLFITVNKKDAKFLNLNLSRGFEKHNFSLEKTVINFENSGIINNTFFNESKKRMPFFG
 FSVNMDFLDTLLACPKIDAEALFNSTSVELTKHMGKSFYKILRSSLASFAQVFDITHNSKFNSCCN
 IYRLGYSMCMRAQAYLKRMDXIFIPQRMFIDTLLNVIGRKILWKKLAEILGYTSRRFLSSAEVKWLC
 LGMRDGLKPSFKYHPCFEQLIYQFQSLTDLIKPLRPVLRQVFLHRRAD

FIG. 52

[illegible]

| | | |
|---------------------|---|-----|
| EST2 pep | FFYCTEISST VTIVYFRHDT WN---- <td>40</td> | 40 |
| Euplotes pep | FFVYTEQQKS YSKTYRKN IWDVI-MKMS IAD-----LKK ETLA--EVOE | 43 |
| Trans of tetrahymen | -----KHKE GSQIFYRKP IWKLVSCLTI VKVRIQFSEK NKQMKNNFYQ | 44 |
| Consensus | FFY.TE..K. .S..YYRK. IW...-KL..-----F..KV.. | 50 |
| EST2 pep | NVCRNHNSY- ----- TLSNFNHSGM RIIPKKSNE FRPIAIPCRG | 79 |
| Euplotes pep | KEVEEWKSL ----- GFAPGKG RIIPKKT-- FRPIPTENKK | 78 |
| Trans of tetrahymen | KIQLEENLE KVEEKLIPED SFQKYPQCKL RIIPKKS-- FRPIPTFLRK | 92 |
| Consensus | K...E.....-----F...CKL RIIPKK...-- FRPIPTF.RK | 100 |
| EST2 pep | ADEEFTIYK ENHKNAIQPT QKILEYLRNK RPTSFTKIYS PTOIADRIKE | 129 |
| Euplotes pep | IVNSDRKTTK LTTWTKLLNS HMLKTLKN- -----RMFK -DPFGFAVFN | 120 |
| Trans of tetrahymen | DKQKNIK--- LNLNQLMDS QLVFRNLKD- -----ML-G -QKIGYSVFD | 130 |
| Consensus |K..K LN.N..L..S QL.L..LKN- -----...IG..VF. | 150 |
| EST2 pep | FKQRLKQFN NVL----- FRPYFMKFD VKSCYD | 157 |
| Euplotes pep | YD-DVMKKQVE EFVCKWKQVH QPKLFFATMD IEKCYD | 155 |
| Trans of tetrahymen | NK-QISEKPA QFIEKWKNG RPKIYVTL- ----- | 158 |
| Consensus | .K....KKF. .F..KWK..G .E..LYF. T.D ...CYD | 186 |

FIG. 53

58/103

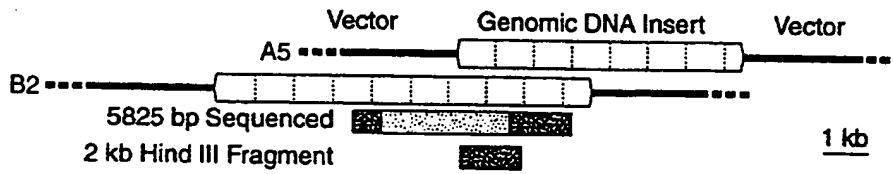


FIG. 55A

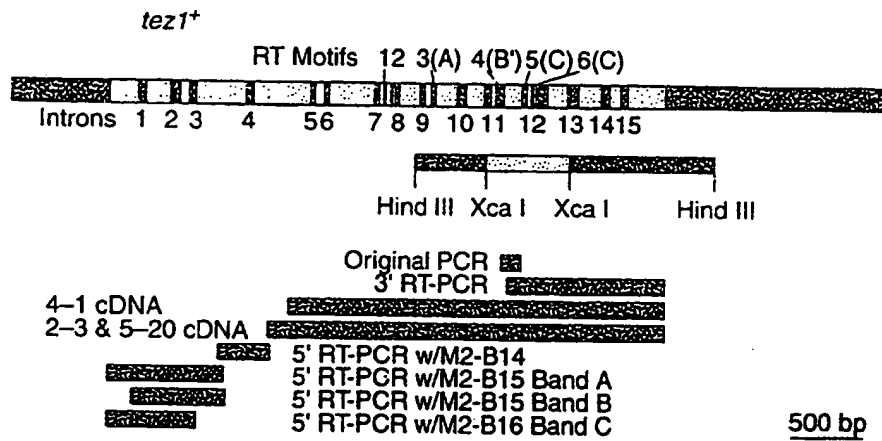


FIG. 55B

59/103

S-1: FFY VTE TTF QKN RLF FYR KSV WSK
S-2: RQH LKR VQL RDV SEA EVR QHR EA
S-3: ART FRR EKR AER LTS RVK ALF SVL NYE

A-1: AKF LHW LMS VYV VEL LRS FFY VTE TTF Q
A-2: LFF YRK SVW SKL QSI GIR QHL KRV QLR DVS
A-3: PAL LTS RLR FIP KPD GLR PIV NMD YVY

FIG. 54

Poly 4

5'- cag acc aaa gga att cca taa gg -3'
Q T K G I P Q G

4(B')

5(c')

3'- ctg ctg atg gag gag tag tgg -5'
a a a a a a a a
t t t t t t
c c
Poly 1

FIG. 56

60/103

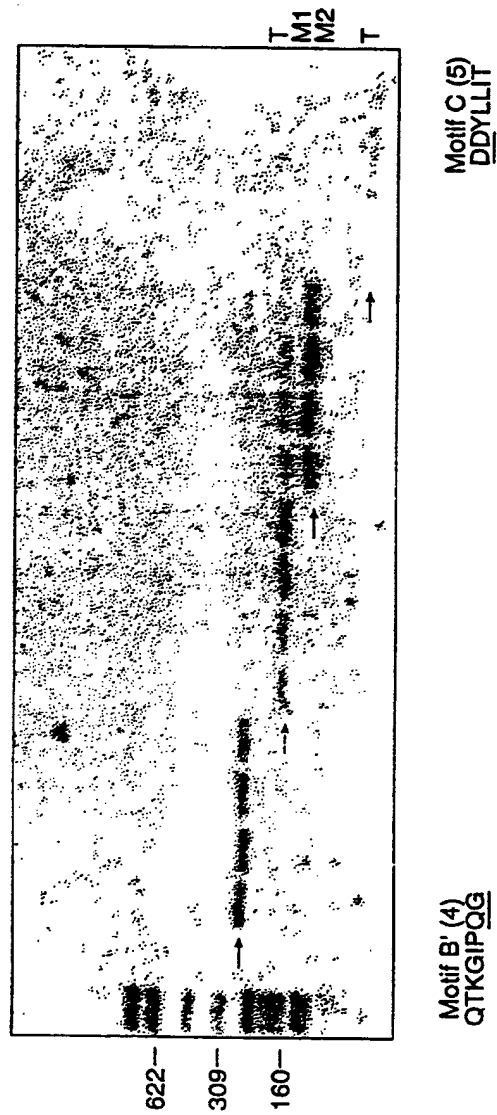


FIG. 57

Ot LCVSYILSSFFYYANLEENALQFLRKESMDPEKPETNLLMRLT
 Ea_p123 KGIPOGLCVSSILSSFFYYATLEESSLGFLRDESMNPENPNVNLMLRLTDDYLLIT
 Sp_M2 SILSSFLCHFYMEDLIDEYLSFTKKK-----GSVLLRVV
 Sc_p103 DGLFQGSLSAPIVDLVYDDLLEFYSEFKASPS-----QDTLILKLADDFLIIS
 * . . . *

Q K V G I P Q G
 caa aaa gtt ggt atc cct cag gg..... <----Actual Genomic Sequence.

Poly 4

t c c
 t a a g c c t c g
 cag acc aaa gga att cca taa gg ---->

ag acc aaa gga att cca tca ggc tca att ctg tca tct ttt ttg tgt cat ttc tat atg
 tc tgg ttt cct taa ggt agt ccg agt taa gac agt aga aaa aac aca gta aag ata tac

K G I P S G S I L S S F L C H F Y M

FIG. 58

62/103

GAA GAT TTG ATT GAT GAA TAC CTA TCG TTT ACG AAA AAG AAA GGA TCA GTG TTG TTA CGA
CTT CTA AAC TAA CTA CTT ATG GAT AGC AAA TGC TTT TTC TTT CCT AGT CAC AAC AAT GCT
E D L I D E Y L S F T K K K G S V L L R

GTA GTC gac gac tac ctc ctc atc acc
CAT CAG ctg ctg atg gag gag tag tgg

V V D D Y L L I T

<---- ctg ctg atg gag gag tag tgg
a a a a a a a
t t t t t
C C
Poly I

.....gac gat ttc ctc ttt ata aca..... <----Actual Genomic Sequence
D D F L F I T

FIG. 58
(CONTINUED)

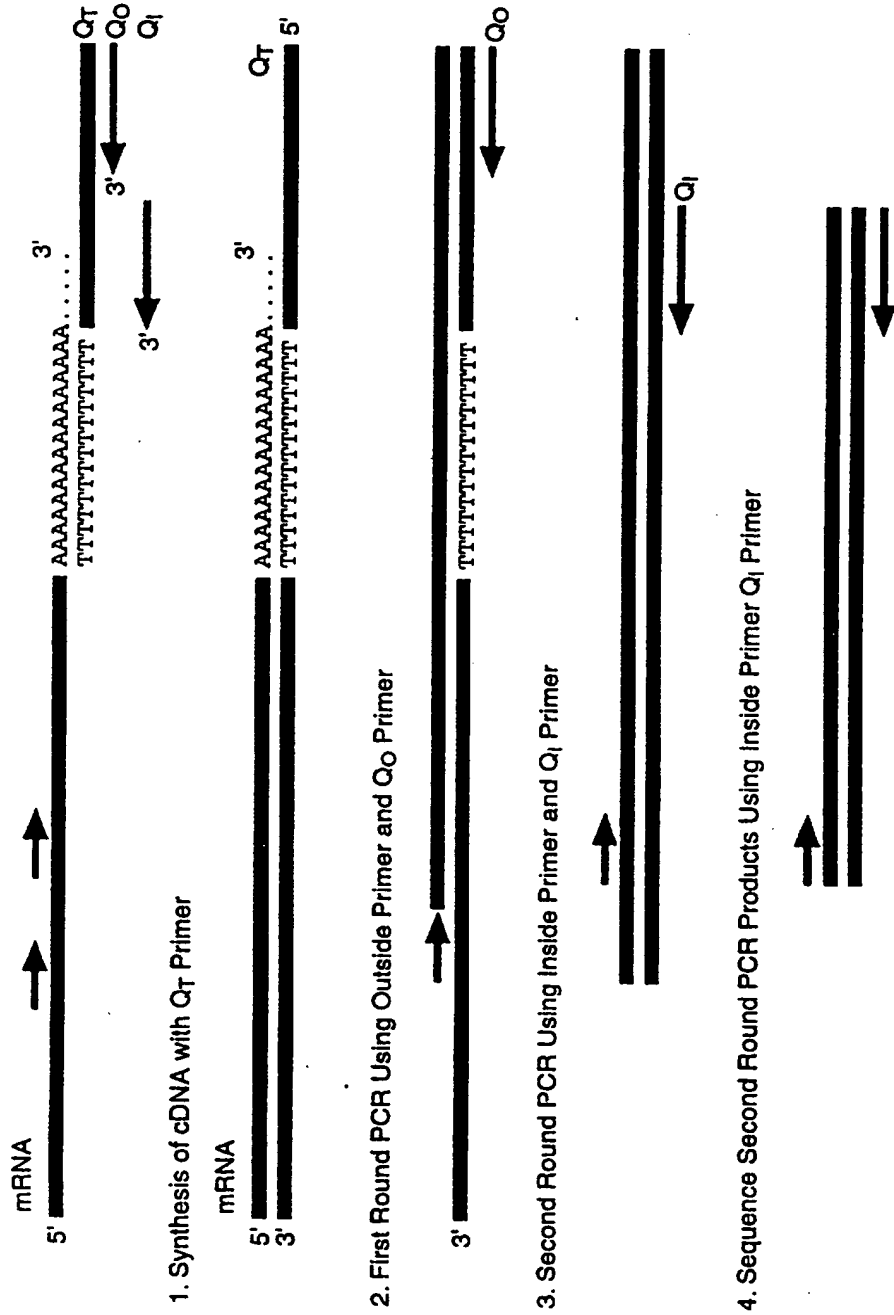


FIG. 59

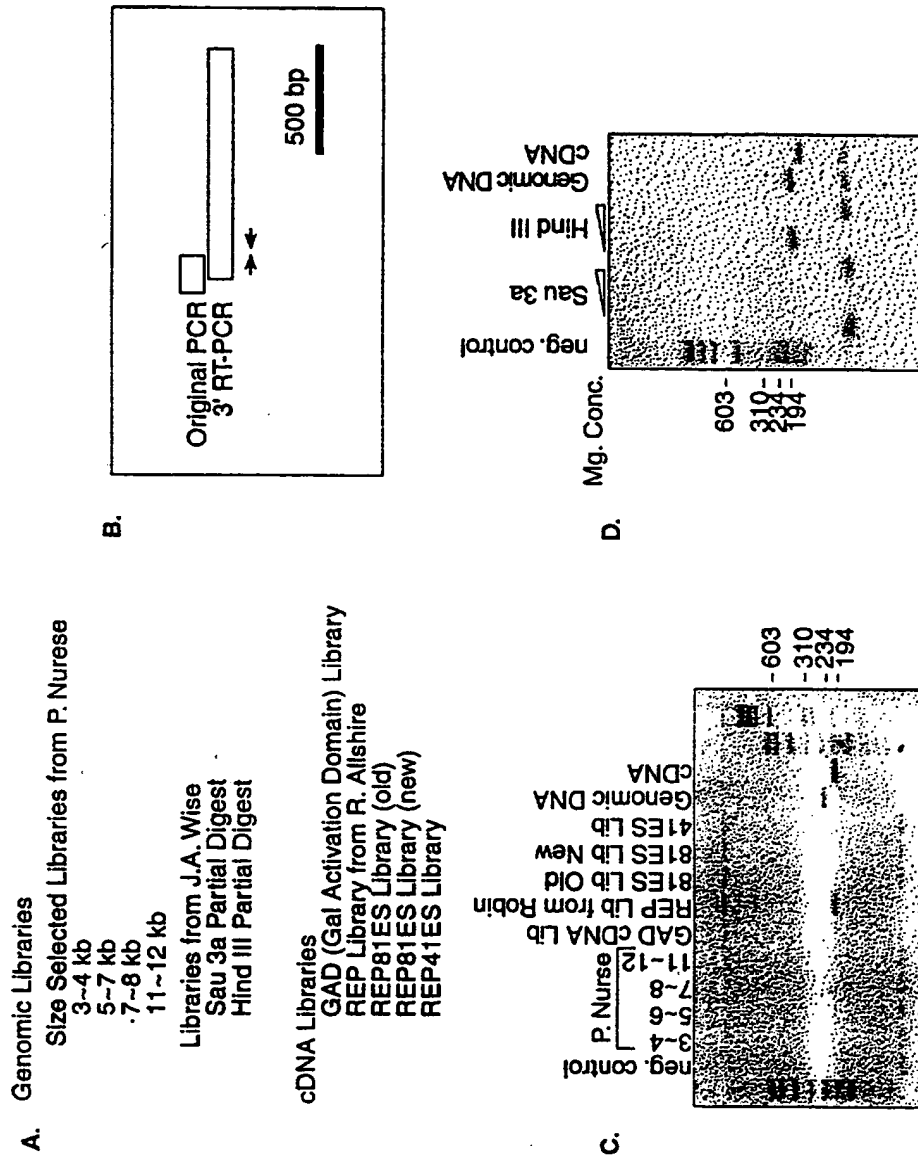


FIG. 60

65/103

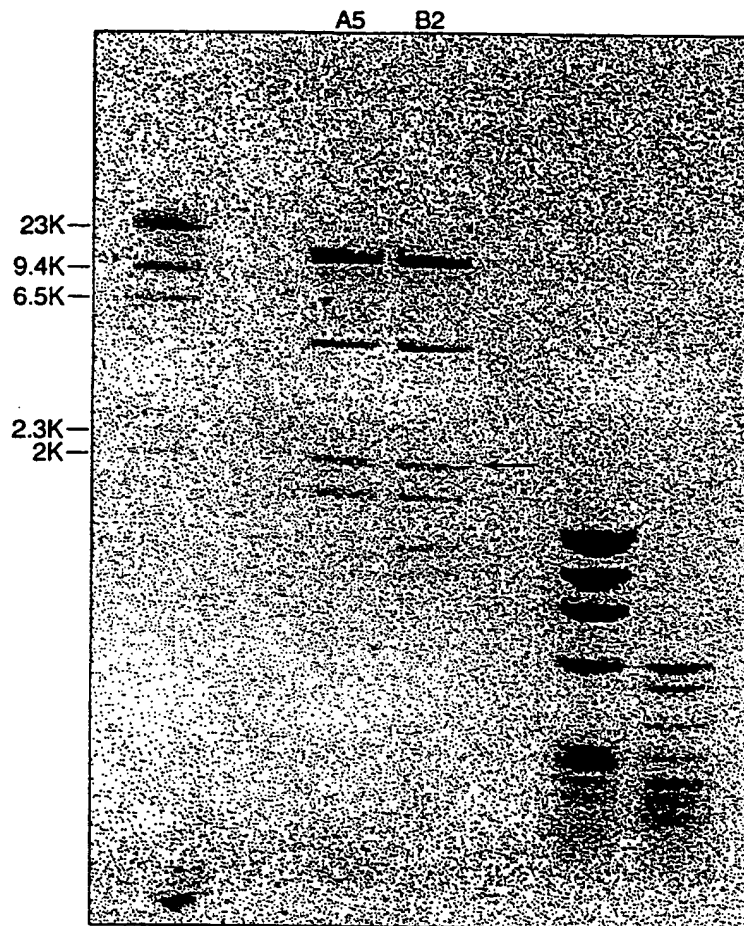


FIG. 61



1. Synthesis of cDNA with Specific Destination Primer



2. Ligate Oligo with 5' -P and blocked 3' to cDNA using T4 RNA Ligase



3. First Round PCR



4. Second Round PCR



FIG. 62

Motif O

S.p. Tez1p (429). WLYNSFIPILOSFYITESSDLNRRTVYFRKDIW ... (35) ...
 S.c. Est2p (366). WLFROLIPKIIQTFFCYCTEISSTVT-IVFRHDTW ... (35) ...
 E.a. p123 (441). WIFEDLVSLIRCFYVTEQQKSYSKTYVRKNIW ... (35) ...
 * * * * *

Motif 1 Motif 2 K

p hh h K hr h R

S.p. Tez1p AVIRLLPKK--NTFRLITN-LRKRF ... (61) ...
 S.c. Est2p SKMRIIPKKSNNFRIIAIPCGAD ... (62) ...
 E.a. p123 GKRLIPKK--TTFRPIMTFNKKIV ... (61) ...
 * * * * *

Motif 3(A) AF

h hDh GY h

S.p. Tez1p KKYFVRIDIKSCYDRIKQDLMFRIK ... (89) ...
 S.c. Est2p ELYFMKFDVKSCYDSIPRMECMRIK ... (75) ...
 E.a. p123 KLFFATMDIEKCYDSVNREKLSTFLK ... (107) ...
 * * * * *

Motif 4(B')

hPQG pP hh h

S.p. Tez1p YLQKVGIPQGSILSSFLCHFYMEDLIDEYLSF ... (6) ...
 S.c. Est2p YIREDFGLFQGSLSAPIVDLVDDLEFYSEF ... (8) ...
 E.a. p123 YKQTKGIPQGLCVSSILSSFPYATLEESSLGF ... (14) ...
 * * * * *

Y Motif 5(C) Motif 6(D)

h F DDhnh Gh h ck h

S.p. Tez1p VLLRVDDFLFITVNNKDAKFLNLSLRGFEKHNFTSLEKTVINFENS ... (205)
 S.c. Est2p LILKLADDDFLIISTDQQQVINIKKLAMGGFKYNKAKNRDKILAVSSQS ... (173)
 E.a. p123 LLMLRTDDYLLITTDQENNAVLFIKLNVSRENGFKFNMKLQTSFPLS ... (209)
 * * * * *

FIG. 63

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|----|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|
| A. | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|----|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|

FIG. 64

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|----|----------|-----|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|-----|-----|-----|---|---|-----|-----|
| A. | Sp_Tip1p | 219 | W | N | S | I | S | R | F | S | I | F | Y | R | S | S | Y | K | K | F | K | Q | D | L | Y | F | N | L | H | S | I | C | D | 251 | | | | | | |
| | Sc_Est2p | 184 | N | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | K | Q | F | L | H | K | L | N | I | N | S | S | S | F | F | P | 200 | | | | | |
| | Ea_p123 | 218 | N | E | K | - | - | D | H | F | L | N | N | I | N | V | P | N | W | N | N | M | K | S | R | T | R | I | F | Y | C | T | H | E | N | 248 | | | | |
| | Sp_Tip1p | 252 | R | N | T | V | H | M | W | L | Q | W | I | F | P | R | Q | F | G | L | I | N | A | F | Q | V | K | Q | L | H | K | V | I | P | L | 284 | | | | |
| | Sc_Est2p | 201 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 223 | | | | | | |
| | Ea_p123 | 249 | R | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 275 | | | | | | |
| | Sp_Tip1p | 285 | V | S | - | - | - | Q | S | T | V | V | P | K | R | L | L | K | V | Y | P | L | I | E | Q | T | A | K | R | L | H | R | I | S | 313 | | | | | |
| | Sc_Est2p | 224 | T | N | - | - | - | - | L | V | K | I | P | Q | R | L | K | V | R | I | N | L | T | L | Q | K | L | L | K | R | H | K | R | L | N | 252 | | | | |
| | Ea_p123 | 276 | F | T | N | I | F | R | F | N | R | I | R | K | K | L | K | D | K | V | I | E | K | I | A | Y | M | L | E | K | V | K | D | F | N | 308 | | | | |
| | Sp_Tip1p | 314 | L | S | K | V | Y | N | H | Y | C | P | Y | I | D | - | T | H | D | D | E | K | I | L | S | Y | S | L | K | P | N | Q | - | - | - | 342 | | | | |
| | Sc_Est2p | 253 | Y | V | S | I | L | N | S | I | C | P | P | L | E | G | T | V | L | D | L | S | H | L | S | R | Q | S | P | K | E | R | - | - | - | 282 | | | | |
| | Ea_p123 | 309 | F | N | Y | Y | L | T | K | S | C | P | L | P | E | N | W | R | E | R | K | Q | I | E | N | L | I | N | K | T | R | E | E | K | 341 | | | | | |
| | Sp_Tip1p | 343 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | V | F | A | F | L | R | S | I | L | V | R | V | F | P | K | L | 359 | |
| | Sc_Est2p | 283 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | V | L | K | F | I | I | V | L | L | Q | K | L | L | P | Q | E | M | 299 |
| | Ea_p123 | 342 | S | K | Y | E | E | L | F | S | Y | T | T | D | N | K | C | V | T | Q | F | I | N | E | F | F | Y | N | I | L | P | K | D | F | 374 | | | | | |
| | Sp_Tip1p | 360 | W | G | N | Q | R | I | F | E | I | I | L | K | D | L | E | T | F | L | K | L | S | R | Y | E | S | F | S | L | H | Y | L | M | S | 392 | | | | |
| | Sc_Est2p | 300 | F | G | S | K | K | N | K | G | K | I | I | K | N | L | N | L | L | S | L | P | L | N | G | Y | L | P | F | D | S | L | L | K | 332 | | | | | |
| | Ea_p123 | 375 | L | T | G | - | R | N | R | K | N | F | Q | K | K | V | K | Y | V | E | L | N | K | H | E | L | I | H | K | N | L | L | E | 408 | | | | | | |
| | Sp_Tip1p | 393 | N | I | K | I | S | E | I | E | W | L | V | L | G | K | R | S | N | A | K | M | C | L | S | D | F | E | K | R | K | Q | I | F | A | 425 | | | | |
| | Sc_Est2p | 333 | K | L | R | L | K | D | F | R | W | L | F | I | S | - | - | D | I | W | F | T | K | H | N | F | E | N | L | N | Q | L | A | I | 362 | | | | | |
| | Ea_p123 | 407 | K | I | N | T | R | E | I | S | W | M | Q | V | E | T | S | - | A | K | H | E | Y | Y | F | D | H | E | N | - | I | Y | V | L | W | 437 | | | | |

FIG. 64
(CONTINUED)

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|----|----------|-----|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|-----|-----|-----|
| A. | Sp_Tip1p | 426 | E | F | I | Y | W | L | Y | N | S | F | I | I | P | I | L | Q | S | F | F | Y | I | T | E | S | S | D | L | R | N | R | I | T | V | Y | 458 |
| | Sc_Est2p | 363 | C | F | I | S | W | L | F | R | Q | L | I | P | K | I | I | Q | T | F | F | Y | C | T | E | I | S | S | T | V | T | - | I | V | Y | 394 | |
| | Ea_p123 | 438 | K | L | L | R | W | I | E | E | D | L | V | V | S | L | I | R | C | F | F | Y | V | T | E | Q | Q | K | S | Y | S | K | T | Y | Y | 470 | |
| | Sp_Tip1p | 459 | F | R | K | D | I | W | K | L | L | C | R | P | F | I | T | S | M | K | M | E | A | F | E | K | I | N | E | N | N | V | R | M | D | 491 | |
| | Sc_Est2p | 395 | F | R | H | D | I | T | W | N | K | L | I | T | P | E | I | V | E | Y | E | K | T | Y | L | V | E | N | N | V | C | R | N | H | N | S | 427 |
| | Ea_p123 | 471 | Y | R | K | N | I | W | D | V | I | M | K | M | S | I | A | D | L | K | K | E | T | L | A | E | V | Q | E | K | E | V | E | E | W | 503 | |
| | Sp_Tip1p | 492 | T | Q | K | T | T | L | P | A | V | I | R | L | L | P | K | K | - | - | N | T | F | R | L | I | T | N | L | R | K | R | F | L | 522 | | |
| | Sc_Est2p | 428 | Y | T | L | S | N | F | N | H | S | K | M | R | I | I | P | K | K | S | N | N | E | F | R | I | I | A | I | P | C | R | G | A | D | 460 | |
| | Ea_p123 | 504 | K | K | S | L | G | F | A | P | G | K | L | R | L | I | P | K | K | - | - | T | T | F | R | P | I | M | T | F | N | K | I | V | 534 | | |
| | Sp_Tip1p | 523 | I | K | M | G | S | N | K | K | M | L | V | S | T | N | Q | T | L | R | P | V | A | S | I | L | K | H | L | I | N | E | - | - | 552 | | |
| | Sc_Est2p | 461 | E | E | E | - | - | F | T | I | Y | K | E | N | H | K | N | A | I | Q | P | T | Q | K | I | L | L | E | Y | L | R | N | K | R | P | T | 491 |
| | Ea_p123 | 535 | N | S | D | - | - | R | K | T | T | K | L | T | N | T | K | L | L | N | S | H | L | M | L | K | T | L | K | N | R | - | M | F | 564 | | |
| | Sp_Tip1p | 553 | E | S | S | G | I | P | F | N | L | E | V | Y | M | K | L | L | T | F | K | K | D | L | L | K | H | R | M | F | G | R | - | K | K | 584 | |
| | Sc_Est2p | 492 | S | F | T | K | I | Y | S | P | T | Q | I | A | D | R | I | K | E | F | K | Q | R | L | L | K | K | F | N | N | V | L | P | E | L | 524 | |
| | Ea_p123 | 565 | K | D | P | F | G | F | A | V | F | N | Y | D | D | V | M | K | K | Y | E | E | F | V | C | K | W | K | Q | V | G | P | K | L | 597 | | |
| | Sp_Tip1p | 585 | Y | F | V | R | I | D | I | K | S | C | Y | D | R | I | K | Q | D | L | M | F | R | I | V | K | K | K | L | K | D | P | E | - | F | 616 | |
| | Sc_Est2p | 525 | Y | F | M | K | F | D | V | K | S | C | Y | D | S | I | P | R | M | E | C | M | R | I | L | K | D | A | L | K | N | E | N | G | F | 557 | |
| | Ea_p123 | 598 | F | F | A | T | M | D | I | E | K | C | Y | D | S | V | N | R | E | K | L | S | T | F | L | K | T | T | K | L | L | S | S | D | E | 630 | |
| | Sp_Tip1p | 617 | V | L | R | K | Y | A | T | I | H | A | T | S | D | R | A | T | K | N | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 634 | |
| | Sc_Est2p | 558 | F | V | R | S | Q | Y | F | F | N | T | N | T | G | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 570 | | |
| | Ea_p123 | 631 | W | I | M | T | A | Q | I | L | K | R | K | N | I | V | I | D | S | K | N | F | R | K | K | E | M | K | D | Y | F | R | Q | K | 663 | | |

FIG. 64
(CONTINUED)

A.

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|----------|-----|------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|-----|---|---|---|-----|-----|-----|-----|
| Sp_Tip1p | 635 | [F]VSE[A]F | S | Y | F | D | M | V | P | E | E | K | V | V | Q | L | S | - | - | M | K | T | S | D | I | L | F | V | 685 | | | | | | | |
| Sc_Est2p | 571 | - | - | - | - | - | - | - | - | - | - | - | V | L | K | L | F | N | V | V | N | A | S | R | - | - | V | P | K | P | Y | E | L | Y | 591 | |
| Ea_p123 | 664 | [F]Q | K | I | A | L | E | G | G | Q | Y | P | T | L | F | S | V | L | E | N | E | Q | N | D | L | N | A | K | K | I | T | L | I | V | 696 | |
| Sp_Tip1p | 686 | [D]F | V | D | Y | W | T | K | S | S | S | E | I | F | K | M | L | K | E | H | L | S | G | H | I | V | K | I | G | N | S | Q | Y | 698 | | |
| Sc_Est2p | 592 | [D]N | V | R | T | V | H | L | S | N | Q | D | V | I | N | V | V | E | M | E | I | F | K | T | A | L | W | V | E | D | K | C | Y | 624 | | |
| Ea_p123 | 697 | E | A | K | Q | R | N | Y | F | K | K | D | N | L | L | Q | P | V | I | N | I | C | Q | Y | N | I | N | F | N | G | K | F | Y | 729 | | |
| Sp_Tip1p | 699 | L | Q | K | V | G | I | P | Q | G | S | I | L | S | S | F | L | C | H | F | Y | M | E | D | L | I | D | E | Y | L | S | F | T | K | 731 | |
| Sc_Est2p | 625 | I | R | E | D | G | L | E | Q | G | S | S | L | S | A | P | I | V | D | L | V | Y | D | D | L | L | E | F | Y | S | E | F | K | A | 657 | |
| Ea_p123 | 730 | K | Q | T | K | G | I | P | Q | G | L | C | V | S | I | L | S | S | F | Y | Y | A | T | L | E | E | S | S | L | G | F | L | R | 762 | | |
| Sp_Tip1p | 732 | K | K | G | - | - | - | - | - | - | - | - | S | V | L | L | R | V | V | D | D | F | L | F | I | T | V | N | K | K | D | A | K | K | 756 | |
| Sc_Est2p | 658 | S | P | S | Q | D | - | - | - | - | - | - | T | L | I | L | K | L | A | D | D | E | L | I | I | S | T | D | Q | Q | Y | I | N | 684 | | |
| Ea_p123 | 763 | D | E | S | M | N | P | E | N | P | N | V | N | L | L | M | R | L | T | D | D | Y | L | L | I | T | T | Q | E | N | N | A | V | L | 795 | |
| Sp_Tip1p | 757 | [E]L | N | L | S | L | R | G | F | E | K | H | N | F | I | S | T | S | L | E | K | T | V | I | N | F | I | E | N | S | I | N | G | - | - | 786 |
| Sc_Est2p | 685 | I | K | K | L | A | M | G | G | E | Q | K | Y | N | A | K | A | N | R | D | K | I | L | A | V | S | S | Q | S | D | - | - | - | - | 713 | |
| Ea_p123 | 796 | [F]I | E | K | L | I | N | V | S | R | E | N | G | E | K | F | N | M | K | K | L | Q | T | S | E | P | L | S | P | S | K | F | A | 828 | | |
| Sp_Tip1p | 787 | - | - | - | I | I | N | N | T | F | F | N | E | S | K | K | R | M | P | F | G | F | S | V | N | M | R | S | L | D | T | L | L | 816 | | |
| Sc_Est2p | 714 | - | - | - | D | D | T | V | I | Q | F | C | A | - | - | M | H | I | F | V | K | E | L | E | V | W | K | H | S | S | T | M | 739 | | | |
| Ea_p123 | 829 | K | Y | G | M | D | S | V | E | E | Q | N | I | V | Q | D | Y | C | D | W | I | G | I | S | I | D | M | K | T | L | A | L | M | P | 861 | |
| Sp_Tip1p | 817 | A | C | P | K | I | D | E | A | L | F | N | S | T | S | V | E | L | T | K | H | M | G | K | S | F | F | Y | K | I | L | R | S | S | 849 | |
| Sc_Est2p | 740 | N | N | F | H | I | R | S | K | S | S | K | G | I | F | R | S | L | I | A | L | F | N | T | R | I | S | Y | K | T | I | D | I | N | 772 | |
| Ea_p123 | 862 | N | I | N | L | R | I | E | G | I | L | C | T | L | N | L | N | M | Q | T | K | K | A | S | M | W | L | K | K | L | K | S | F | 894 | | |

FIG. 64
(CONTINUED)

| | | | |
|----------|------|---|------|
| A. | | | |
| Sp_Tip1p | 850 | L A S F A Q V F I D I T H N S K F N S C C N I Y R L G Y S M C M R | 882 |
| Sc_Est2p | 773 | L N S T N T V L M Q I D H V V K N I S E C | 793 |
| Ea_p123 | 895 | L M N N I T H Y F R K T I T T E D F A N K T L N K L F I S G G Y K | 927 |
| Sp_Tip1p | 883 | A Q A Y L K R M K D I E I P Q R M F I T D L L N V I G R K I W K K | 915 |
| Sc_Est2p | 794 | - - - Y K S A F K D L S I N - - V T Q N M Q F H S F L Q R I I E M | 821 |
| Ea_p123 | 928 | Y M Q C A K E Y K D H F E K K N L A M S S M I D L E V S K I I Y S V | 960 |
| Sp_Tip1p | 916 | L A E I L G Y T S R R F L S S A E V K W L F C L G M R D G L K P S | 948 |
| Sc_Est2p | 822 | T V S G C P I I T K C D P L I E Y E V R F T I L N G F L E S L S S N | 854 |
| Ea_p123 | 961 | T R A F F K Y L V C N I K D T I F G E E H Y P D F F L S T L K H F | 983 |
| Sp_Tip1p | 949 | F K Y H P C F E Q L I Y Q F Q S L T D L I K P L R P V L R Q V L F | 981 |
| Sc_Est2p | 855 | T S - - - - - K F K D N I I L L R K E I Q H L Q A Y I Y | 877 |
| Ea_p123 | 994 | I E I F S - - - T K K Y I E N R V C M I L K A K E A K L K S D Q C | 1023 |
| Sp_Tip1p | 982 | L H R R I A D - | 988 |
| Sc_Est2p | 878 | I Y I H I V N - | 884 |
| Ea_p123 | 1024 | Q S L I Q Y D A | 1031 |

FIG. 64
(CONTINUED)

B.

| | | | | |
|----------|-----|-----------------------|----------------------|-----|
| Sp_Tip1p | 1 | -----MTEHHTPKSRILRFL | ENQYVYLCT | 24 |
| Sc_Est2p | 1 | ----- | -----MKILFEF | 7 |
| Ea_p123 | 1 | MEVDVDNQADNHGHSALKTC | EEIKEAKTLYSW | 33 |
| Sp_Tip1p | 25 | LNDYVQLVLRGSPAS | YSNICERLRSDVQTSFS | 57 |
| Sc_Est2p | 8 | IQDKLDIDLQTN-- | STYK--ENLKCGHFNGLD | 35 |
| Ea_p123 | 34 | IQKVIRCRNQSQ-- | SHYK--DLEDIKIFAQTN | 61 |
| Sp_Tip1p | 58 | IFLHSTVVGFDSPDEGVQFSS | PKCSQSEL | 90 |
| Sc_Est2p | 36 | EILTTTCFALPNSR-KIAL | PCLPGLSHKAV | 67 |
| Ea_p123 | 62 | IVATPRDYNEEDFKVIARKE | VFSTGLMIELIDK | 94 |
| Sp_Tip1p | 91 | VVKQMFDESFERRR-NLL | MKGFSMNHEDFRAMH | 122 |
| Sc_Est2p | 68 | CIYLLLTGELYN-- | NVLTFGYKARNED | 93 |
| Ea_p123 | 95 | CLVELLSSSDVSDRQKLQCF | GFQLKGNQ | 122 |
| Sp_Tip1p | 123 | VNGVQNDLVSTFPNYLIS | LESKNWQLLLEII | 155 |
| Sc_Est2p | 94 | --VNNSLFCHSANVNVT | LLKGAAMKMFHSLVG | 123 |
| Ea_p123 | 123 | --LAKTHLLTALSTQKQY | FFQDEWNQVRAMIG | 152 |
| Sp_Tip1p | 156 | SDAMHYLLLSKGSIFEAL | PNDNYLQISG | 188 |
| Sc_Est2p | 124 | TYAFVDLLINVTVIQFN- | GQFFTQIVGNRCNEP | 155 |
| Ea_p123 | 153 | NELFRHLLYTKYLI | FQRTSEGTLVQFCGNNVFDH | 185 |
| Sp_Tip1p | 189 | NVFEETVSKKRKRTIETS | ITQN--KSARKEVS | 218 |
| Sc_Est2p | 156 | HLPPKWVQ--RSSSSSA | TAAQI--KQLTEPVT | 183 |
| Ea_p123 | 186 | LKVNDKFDK-KQKGGAA | ADMNEPRCCSTCKYNVK | 217 |

FIG. 64
(CONTINUED)

B.

| | | | | | | | | |
|----------|-----|--------------|-------|--------|-------|-------|-------|-----|
| Sp_Tip1p | 219 | WNSISISRFSIF | YRSS | YKFKQD | LYFN | LH | SICD | 251 |
| Sc_Est2p | 184 | N----- | ----- | KQFL | HK | LN | INSS | 200 |
| Ea_p123 | 218 | NEK--DHFL | NNIN | VPNW | NNMK | SR | TRIFY | 248 |
| Sp_Tip1p | 252 | RNTVHMW | LQWIF | PRQF | GLIN | AFQV | KQLH | 284 |
| Sc_Est2p | 201 | ----- | YSKIL | PSS-- | --S | IKKL | TDL | 223 |
| Ea_p123 | 249 | R----- | NNQF | FKH | EFV | SN | NNIS | 275 |
| Sp_Tip1p | 285 | VS----- | QSTV | VVPK | RLLK | VYPL | IEQT | 313 |
| Sc_Est2p | 224 | TN----- | LVKI | PQRL | KVRIN | LTQL | KLKR | 252 |
| Ea_p123 | 276 | FTNIFR | FNRI | RKKL | KDKV | IEKIA | YMLE | 308 |
| Sp_Tip1p | 314 | LSKVYNH | YC | PYID | -THD | EKIL | SYSL | 342 |
| Sc_Est2p | 283 | YVSI | LN | SI | CP | PLEG | TVLD | 282 |
| Ea_p123 | 309 | FNYYL | TK | SC | P | LPEN | WRER | 341 |
| Sp_Tip1p | 343 | ----- | ----- | ----- | ----- | VFA | FLRS | 359 |
| Sc_Est2p | 283 | ----- | ----- | ----- | ----- | VLF | FI | 299 |
| Ea_p123 | 342 | SKYYE | ELFS | YTT | DN | KCV | TQ | 374 |
| Sp_Tip1p | 360 | WGNQR | I | FEI | I | LDLE | TF | 392 |
| Sc_Est2p | 300 | FGSK | KN | KG | KI | KN | LN | 332 |
| Ea_p123 | 375 | LTG-R | NR | KN | FQ | KV | KY | 406 |
| Sp_Tip1p | 393 | NIKISE | I | EW | LV | L | GK | 425 |
| Sc_Est2p | 333 | KLRL | KD | FR | W | L | FIS | 382 |
| Ea_p123 | 407 | KINTRE | I | S | WM | Q | VETS | 437 |

FIG. 64

(CONTINUED)

B.

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|----------|-----|----|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|-----|---|-----|-----|
| Sp_Tip1p | 426 | EF | I | W | L | Y | N | S | F | I | P | I | L | Q | S | F | F | Y | I | T | E | S | S | D | L | R | N | R | T | V | Y | 458 | | | |
| Sc_Est2p | 363 | C | F | I | S | W | L | F | R | Q | L | I | P | K | I | I | Q | T | F | F | Y | C | T | E | I | S | S | T | V | T | - | I | V | Y | 394 |
| Ea_p123 | 438 | K | L | L | R | W | I | F | E | D | L | V | V | S | L | I | R | C | F | F | Y | V | T | E | Q | Q | K | S | Y | S | K | T | Y | 470 | |
| Sp_Tip1p | 459 | F | R | K | D | I | W | K | L | L | C | R | P | F | I | T | S | M | K | M | E | A | F | E | K | I | N | E | N | N | V | R | M | D | 491 |
| Sc_Est2p | 395 | F | R | H | D | T | W | N | K | L | I | T | P | F | I | V | E | Y | F | K | T | Y | L | V | E | N | N | V | C | R | N | H | N | S | 427 |
| Ea_p123 | 471 | Y | R | K | N | I | W | D | V | I | M | K | M | S | I | A | D | L | K | K | E | T | L | A | E | V | Q | E | K | E | V | E | E | W | 503 |
| Sp_Tip1p | 492 | T | Q | K | T | T | L | P | P | A | V | I | R | L | L | P | K | K | - | - | N | T | F | R | L | I | T | N | L | R | K | R | F | L | 522 |
| Sc_Est2p | 428 | Y | T | L | S | N | F | N | H | S | K | M | R | I | I | P | K | K | S | N | N | E | F | R | I | I | A | I | P | C | R | G | A | D | 460 |
| Ea_p123 | 504 | K | K | S | L | G | F | A | P | G | K | L | R | L | I | P | K | K | - | - | T | T | F | R | P | I | M | T | F | N | K | K | I | V | 534 |
| Sp_Tip1p | 523 | I | K | M | G | S | N | K | K | M | L | V | S | T | N | Q | T | L | R | P | V | A | S | I | L | K | H | L | I | N | E | - | - | - | 552 |
| Sc_Est2p | 461 | E | E | E | - | - | F | T | I | Y | K | E | N | H | K | N | A | I | Q | P | T | Q | K | I | L | E | Y | L | R | N | K | R | P | T | 491 |
| Ea_p123 | 535 | N | S | D | - | - | R | K | T | T | K | L | T | T | N | T | K | L | L | N | S | H | L | M | L | K | T | L | K | N | R | - | M | F | 564 |
| Sp_Tip1p | 553 | E | S | S | G | I | P | F | N | L | E | V | Y | M | K | L | L | T | F | K | K | D | L | L | K | H | R | M | F | G | R | - | K | K | 584 |
| Sc_Est2p | 492 | S | F | T | K | I | Y | S | P | T | Q | I | A | D | R | I | K | E | F | K | Q | R | L | L | K | K | F | N | N | V | L | P | E | L | 524 |
| Ea_p123 | 585 | K | D | P | F | G | F | A | V | F | N | Y | D | D | V | M | K | K | Y | E | E | F | V | C | K | W | K | Q | V | G | Q | P | K | L | 597 |
| Sp_Tip1p | 585 | Y | F | V | R | I | D | I | K | S | C | Y | D | R | I | K | Q | D | L | M | F | R | I | V | K | K | L | K | D | P | E | - | F | 616 | |
| Sc_Est2p | 525 | Y | F | M | K | F | D | V | K | S | C | Y | D | S | I | P | R | M | E | C | M | R | I | L | K | D | A | L | K | N | E | N | G | F | 557 |
| Ea_p123 | 598 | F | F | A | T | M | D | I | E | K | C | Y | D | S | V | N | R | E | K | L | S | T | F | L | K | T | T | K | L | L | S | S | D | F | 630 |
| Sp_Tip1p | 617 | V | I | R | K | Y | A | T | I | H | A | T | S | D | R | A | T | K | N | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 634 |
| Sc_Est2p | 558 | F | V | R | S | Q | Y | F | F | N | T | N | T | G | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 570 | |
| Ea_p123 | 631 | W | I | M | T | A | Q | I | L | K | R | K | N | I | V | I | D | S | K | N | F | R | K | K | E | M | K | D | Y | F | R | Q | K | 683 | |

FIG. 64
(CONTINUED)

B.

| | | | | | | | | | |
|----------|-----|------------------|--------|------------|--------|-----------|------------|----|------------|
| Sp_Tip1p | 635 | FVSEAFSYFDMVPFEK | V | VQLLS | -- | MKTS | DTL | FV | 665 |
| Sc_Est2p | 571 | ----- | VLKLFN | VVNASR | -- | VPKPYEL | YI | | 591 |
| Ea_p123 | 604 | FQKIALEGQYPTLFS | V | LENEQN | DLNA | KTKL | IV | | 696 |
| Sp_Tip1p | 666 | DFVDYWTKSSSEI | F | KMLKEHLSGH | I | VKIGNSQ | Y | | 698 |
| Sc_Est2p | 592 | DNVRTVHLSNQDV | I | VVEME | I | FKTALWVED | KCY | | 824 |
| Ea_p123 | 697 | EAKQRNYFKKDN | L | LQPV | I | NCQY | YNFNGKFY | | 729 |
| Sp_Tip1p | 699 | LQKVG | I | PQGS | I | LS | SFLCHFYMED | L | IDEYLS |
| Sc_Est2p | 625 | IREDG | L | FQGS | SSL | S | APIVDLVYDD | L | LEFYSE |
| Ea_p123 | 730 | KQTKG | I | PQGL | CVS | S | ILSSFYAT | L | EESLGLR |
| Sp_Tip1p | 732 | KKG | --- | --- | SVLLRV | DD | FL | F | ITVNKKDAKK |
| Sc_Est2p | 658 | SPSQD | --- | --- | TLILKL | ADD | FL | I | STDQQQVIN |
| Ea_p123 | 763 | DESMNPENPNV | N | LLMRL | T | DD | Y | L | ITTQENNAVL |
| Sp_Tip1p | 757 | FLNLSLRGFEKH | N | FST | S | LEK | TV | IN | FEN |
| Sc_Est2p | 685 | IKKLAMGGFQ | KYN | AKAN | R | D | K | I | LAVSSQSD |
| Ea_p123 | 796 | FIEKLINVSRE | NGFK | FN | M | K | L | Q | T |
| Sp_Tip1p | 787 | --- | I | IN | NTFF | NESK | K | R | M |
| Sc_Est2p | 714 | --- | DD | T | V | I | Q | F | CA |
| Ea_p123 | 829 | KYGMD | S | V | EE | Q | N | I | V |
| Sp_Tip1p | 817 | ACPKIDEAL | F | N | S | T | S | V | E |
| Sc_Est2p | 740 | NNFH | I | R | S | K | S | K | G |
| Ea_p123 | 862 | NINLRI | E | G | I | L | C | T | L |

FIG. 64
(CONTINUED)

78/103

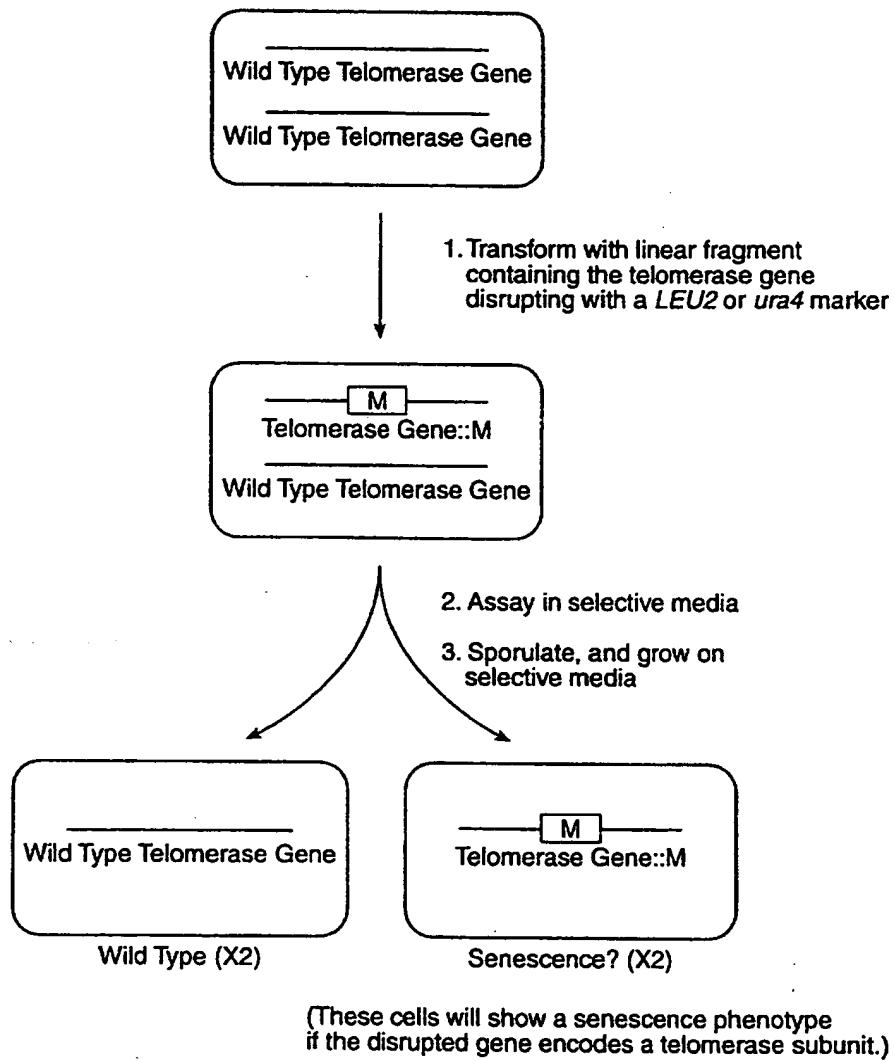
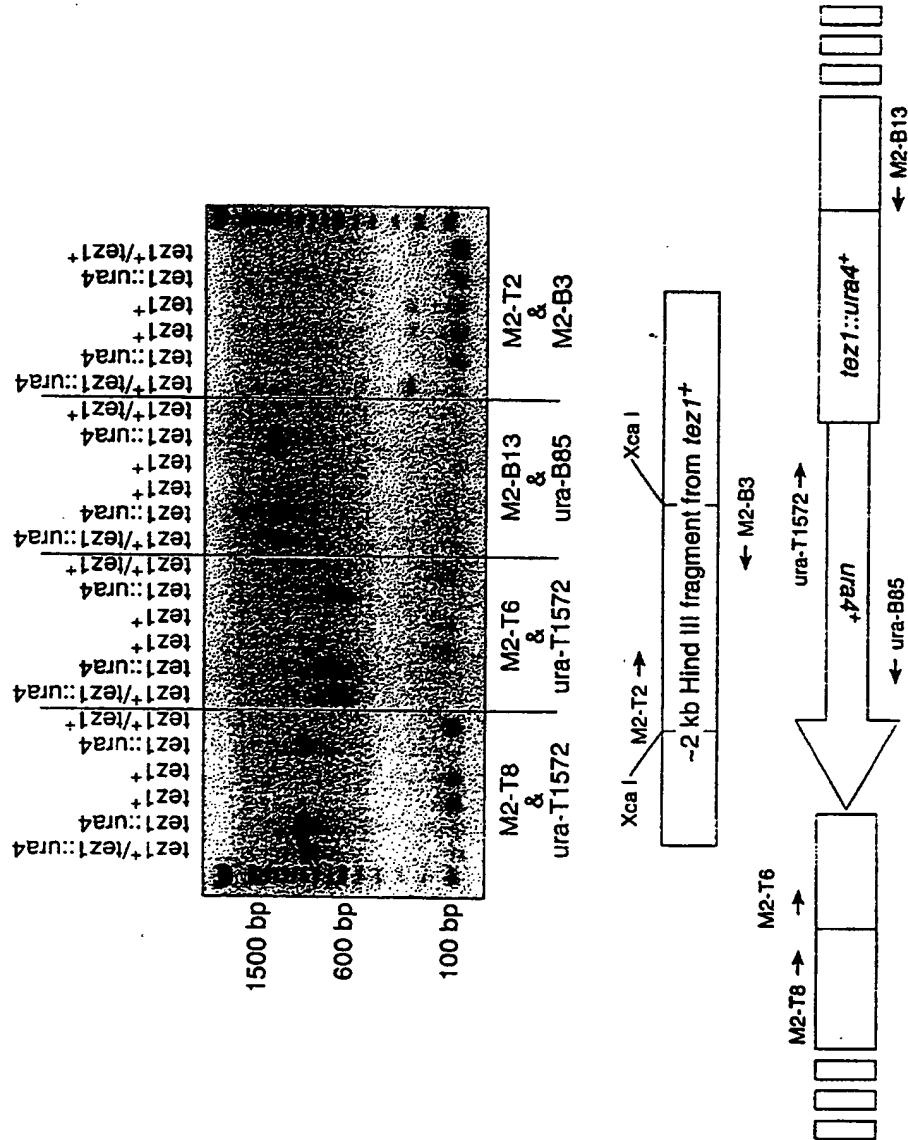


FIG. 65



80/103

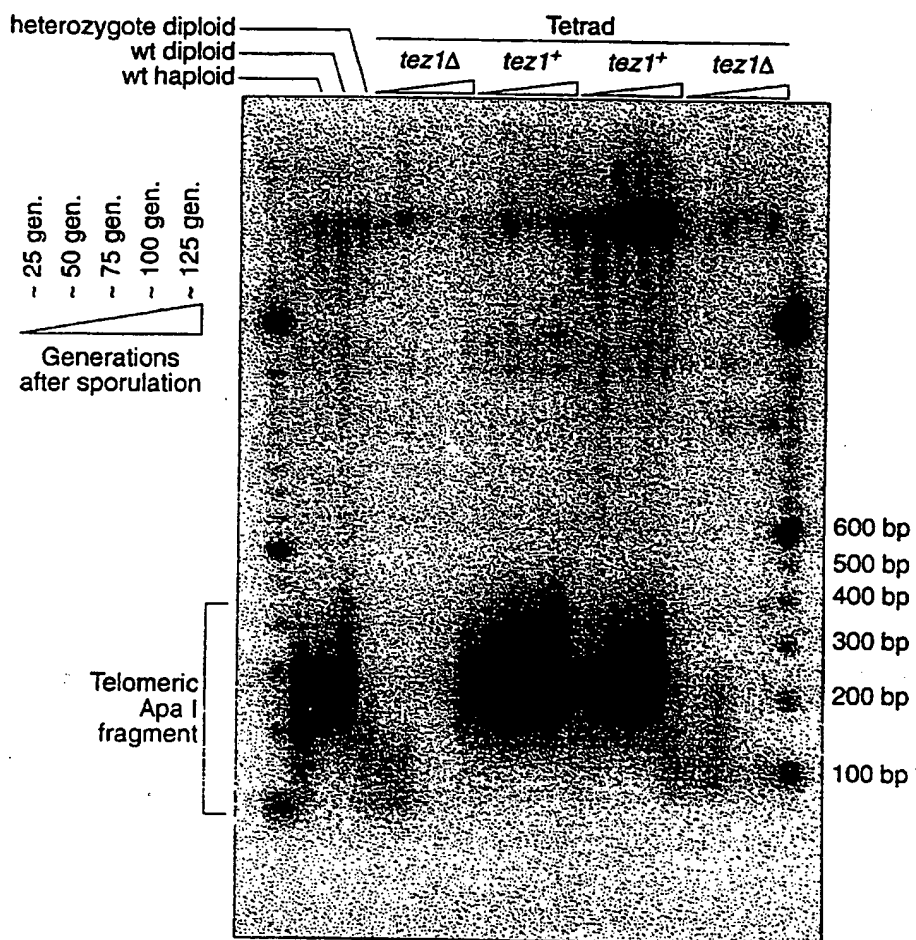


FIG. 67

81/103

```

      1
GCCAAGTTCCTGCACTGGCTG  met ser val tyr val val glu leu leu
      ATG AGT GTG TAC GTC GTC GAG CTG CTC

10      20
arg ser phe phe tyr val thr glu thr thr phe gln lys asn arg
AGG TCT TTC TTT TAT GTC ACG GAG ACC ACG TTT CAA AAG AAC AGG

      30
leu phe phe tyr arg lys ser val trp ser lys leu gln ser ile
CTC TTT TTC TAC CGG AAG AGT GTC TGG AGC AAG TTG CAA AGC ATT

40      50
gly ile arg gln his leu lys arg val gln leu arg glu leu ser
GGA ATC AGA CAG CAC TTG AAG AGG GTG CAG CTG CGG GAG CTG TCG

      60
glu ala glu val arg gln his arg glu ala arg pro ala leu leu
GAA GCA GAG GTC AGG CAG CAT CGG GAA GCC AGG CCC GCC CTG CTG

70      80
thr ser arg leu arg phe ile pro lys pro asp gly leu arg pro
ACG TCC AGA CTC CGC TTC ATC CCC AAG CCT GAC GGG CTG CGG CCG

      90
ile val asn met asp tyr val val gly ala arg thr phe arg arg
ATT GTG AAC ATG GAC TAC GTC GTG GGA GCC AGA ACG TTC CGC AGA

100      110
glu lys ala glu arg leu thr ser arg val lys ala leu phe
GAA AAG ARG GCC GAG CGT CTC ACC TCG AGG GTG AAG GCA CTG TTC

      120
ser val leu asn tyr glu arg ala arg arg pro gly leu leu gly
AGC GTG CTC AAC TAC GAG CGG GCG CGG CGC CCC GGC CTC CTG GGC

130      140
ala ser val leu gly leu asp asp ile his arg ala trp arg thr
GCC TCT GTG CTG GGC CTG GAC GAT ATC CAC AGG GCC TGG CGC ACC

      150
phe val leu arg val arg ala gln asp pro pro pro glu leu tyr
TTC GTG CTG CGT GTG CGG GCC CAG GAC CCG CCG CCT GAG CTG TAC

160      170
phe val lys val asp val thr gly ala tyr asp thr ile pro gln
TTT GTC AAG GTG GAT GTG ACG GGC GCG TAC GAC ACC ATC CCC CAG

      180
asp arg leu thr glu val ile ala ser ile ile lys pro gln asn
GAC AGG CTC ACG GAG GTC ATC GCC AGC ATC ATC AAA CCC CAG AAC

190      200
thr tyr cys val arg arg tyr ala val val gln lys ala ala met
ACG TAC TGC GTG CGT CGG TAT GCC GTG GTC CAG AAG GCC GCC ATG

```

FIG. 68

82/103

210
gly thr ser ala arg pro ser arg ala thr ser tyr val gln cys
GGC ACG TCC GCA AGG CCT TCA AGA GCC ACG TCC TAC GTC CAG TGC

220 230
gln gly ile pro gln gly ser ile leu ser thr leu leu cys ser
CAG GGG ATC CCG CAG GGC TCC ATC CTC TCC ACG CTG CTC TGC AGC

240
leu cys tyr gly asp met glu asn lys leu phe ala gly ile arg
CTG TGC TAC GGC GAC ATG GAG AAC AAG CTG TTT GCG GGG ATT CGG

250 260
arg asp gly leu leu leu arg leu val asp asp phe leu leu val
CGG GAC GGG CTG CTC CTG CGT TTG GTG GAT GAT TTC TTG TTG GTG

270
thr pro his leu thr his ala lys thr phe leu arg thr leu val
ACA CCT CAC CTC ACC CAC GCG AAA ACC TTC CTC AGG ACC CTG GTC

280 290
arg gly val pro glu tyr gly cys val val asn leu arg lys thr
CGA GGT GTC CCT GAG TAT GGC TGC GTG GTG AAC TTG CGG AAG ACA

300
val val asn phe pro val glu asp glu ala leu gly gly thr ala
GTG GTG AAC TTC CCT GTA GAA GAC GAG GCC CTG GGT GGC ACG GCT

310 320
phe val gln met pro ala his gly leu phe pro trp cys gly leu
TTT GTT CAG ATG CCG GCC CAC GGC CTA TTC CCC TGG TGC GGC CTG

330
leu leu asp thr arg thr leu glu val gln ser asp tyr ser ser
CTG CTG GAT ACC CGG ACC CTG GAG GTG CAG AGC GAC TAC TCC AGC

340 350
tyr ala arg thr ser ile arg ala ser leu thr phe asn arg gly
TAT GCC CGG ACC TCC ATC AGA GCC AGT CTC ACC TTC AAC CGC GGC

360
phe lys ala gly arg asn met arg arg lys leu phe gly val leu
TTC AAG GCT GGG AGG AAC ATG CGT CGC AAA CTC TTT GGG GTC TTG

370 380
arg leu lys cys his ser leu phe leu asp leu gln val asn ser
CGG CTG AAG TGT CAC AGC CTG TTT CTG GAT TTG CAG GTG AAC AGC

390
leu gln thr val cys thr asn ile tyr lys ile leu leu leu gln
CTC CAG ACG GTG TGC ACC AAC ATC TAC AAG ATC CTC CTG CTG CAG

400 410
ala tyr arg phe his ala cys val leu gln leu pro phe his gln
GCG TAC AGG TTT CAC GCA TGT GTG CTG CAG CTC CCA TTT CAT CAG

FIG. 68
(CONTINUED)

83/103

420
gln val trp lys asn pro his phe ser cys ala ser ser leu thr
CAA GTT TGG AAG AAC CCA CAT TTT TCC TGC GCG TCA TCT CTG ACA

430 440
arg leu pro leu leu leu his pro glu ser gln glu arg arg asp
CGG CTC CCT CTG CTA CTC CAT CCT GAA AGC CAA GAA CGC AGG GAT

450
val ala gly gly gln gly arg arg arg pro ser ala leu arg gly
GTC GCT GGG GGC CAA GGG CGC CGC CGG CCC TCT GCC CTC CGA GGC

460 470
arg ala val ala val pro pro ser ile pro ala gln ala asp ser
CGT GCA GTG GCT GTG CCA CCA AGC ATT CCT GCT CAA GCT GAC TCG

480
thr pro cys his leu arg ala thr pro gly val thr gln asp ser
ACA CCG TGT CAC CTA CGT GCC ACT CCT GGG GTC ACT CAG GAC AGC

490 500
pro asp ala ala glu ser glu ala pro gly asp asp ala asp cys
CCA GAC GCA GCT GAG TCG GAA GCT CCC GGG GAC GAC GCT GAC TGC

510
pro gly gly arg ser gln pro gly thr ala leu arg leu gln asp
CCT GGA GGC CGC AGC CAA CCC GGC ACT GCC CTC AGA CTT CAA GAC

520 530
his pro gly leu met ala thr arg pro gln pro gly arg glu gln
CAT CCT GGA CTG ATG GCC ACC CGC CCA CAG CCA GGC CGA GAG CAG

540
thr pro ala ala leu ser arg arg ala tyr thr ser gln gly gly
ACA CCA GCA GCC CTG TCA CGC CGG GCT TAT ACG TCC CAG GGA GGG

550 560
arg gly gly pro his pro gly leu his arg trp glu ser glu ala
AGG GGC GGC CCA CAC CCA GGC CTG CAC CGC TGG GAG TCT GAG GCC

564
OP
TGA GTGAGTGTGTTGGCCGAGGCCTGCATGTCCGGCTGAAGGCTGAGTGTCCGGCTGAGGC
CTGAGCGAGTGTCCAGCCAAGGGCTGAGTGTCCAGCACACCTGCGTTTTCACTTCCCCAC
AGGCTGGCGTTTCGGTCCACCCAGGGCCAGCTTTTCCTCACCAGGAGCCCGGCTTCCACT
CCCCACATAGGAATAGTCCATCCCCAGATTGCGCCATTGTTACCCCTTCGCCCTGCCTTCC
TTTGCCCTTCCACCCCCACCATTCAGGTGGAGACCCTGAGAAGGACCCTGGGAGCTTTGGG
AATTTGGAGTGACCAAAGGTGTGCCCTGTACACAGGCGAGGACCCTGCACCTGGATGGGG
GTCCCTGTGGGTCAAATTGGGGGGAGGTGCTGTGGGAGTAAATACTGAATATATGAGTT
TTTCAGTTTTTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

FIG. 68
(CONTINUED)

| | |
|-----------|---|
| Motif -1 | |
| Ep p123 | ...LVVSLIRCFFYVTEQQSYSKT... |
| Sp Tez1 | ...FIIPILQSFFYITESDLRNRT... |
| Sc Est2 | ...LIPKIIQTFFYCTEISSTVTIV... |
| Hs TCP1 | ...YVVELLRSFFYVTETTFQKNRL... |
| consensus | FFY TE |
| | |
| Motif 0 | p hhh K hR h K |
| Ep p123 | ...KSLGFAPGKLRLLIPKKT--TFRPIMTFNKKIV... |
| Sp Tez1 | ...QKTTLPPAVIRLLPKKN--TFLRLITNLRKRFL... |
| Sc Est2 | ...TLSNFMHNSKMRIIPKSNNEFRIIAIPCRGAD... |
| Hs TCP1 | ...ARPALLTSRLRFIPKPD--GLRPVNMMDYVVG... |
| consensus | R PK R I |
| | |
| Motif A | AF |
| | h hDh GY h |
| Ep p123 | ...PKLFFATMDIEKCYDSVNREKLSTFLK... |
| Sp Tez1 | ...RKKYFVRIDIKSCYDRIKQDLMFRIVK... |
| Sc Est2 | ...PELYFMKFVDKSCYDSIPRMECMRILK... |
| Hs TCP1 | ...PELYFVKVDVTGAYDTIPQDRLTEVIA...//... |
| consensus | F D YD |
| | |
| Motif B | hPQG pS hh |
| Ep p123 | ...NGKFYKQTKGIPQGLCVSSILSSFYYA... |
| Sp Tez1 | ...GNSQYLQKVGIPQGSILSSFLCHFYME... |
| Sc Est2 | ...EDKCYIREDGLFQGSLSAPIVDLVYD... |
| Hs TCP1 | ...RATSYVQCQGIPQGSILSTLLCSLCYG... |
| consensus | G QG S |
| | |
| Motif C | Y |
| | h F DD hhh |
| Ep p123 | ...PNVNLLMRLTDDYLLITTQENN... |
| Sp Tez1 | ...KKGSVLLRVDDFLFITVNKKD... |
| Sc Est2 | ...SQDTLILKLADDFLIISTDQQQ... |
| Hs TCP1 | ...RRDGLLLRLVDDFLLVTPHLTH... |
| consensus | DD L |
| | |
| Motif D | Gh h cK |
| Ep p123 | ...NVSRENGFKFNMKKL... |
| Sp Tez1 | ...LNLSLRGFEKHNFT... |
| Sc Est2 | ...KKLAMGGFQKYNKA... |
| Hs TCP1 | ...LRTLVRGVPEYGCVV... |
| consensus | G |

FIG. 69

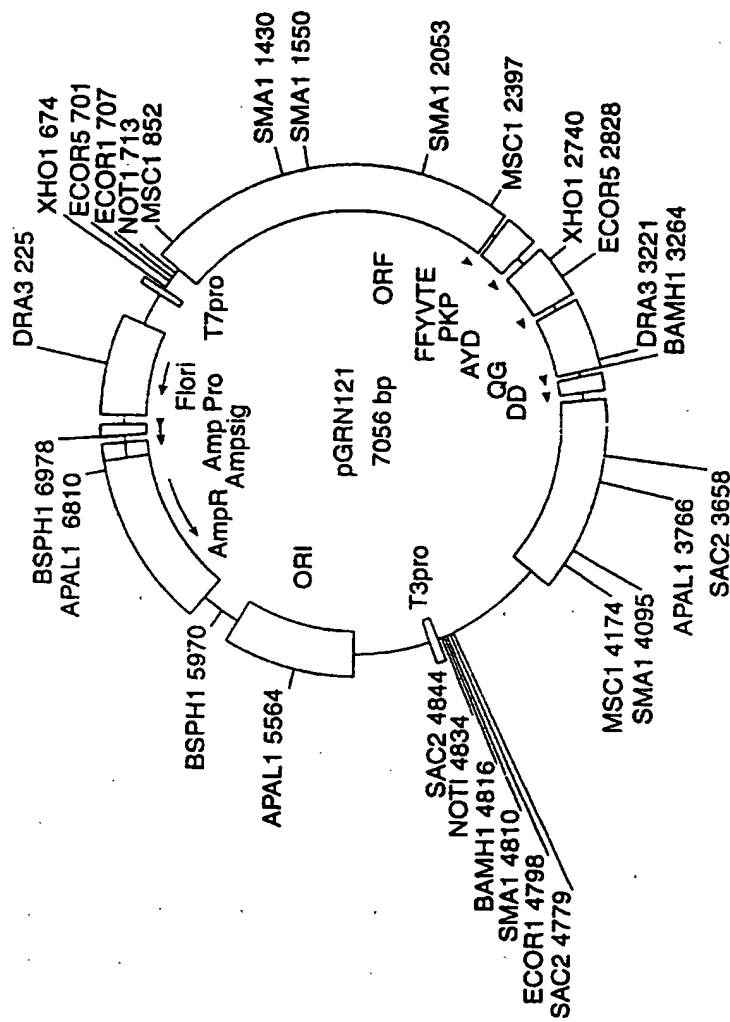


FIG. 70

```

1   GCACGCGTGC GTCTGTCTGC GCACGTGGGA AGCCCTGGCC CCGGCCACCC
51  CCGCGATGCC GCGCGCTCCC CGCTGCCGAG CCGTGCGCTC CCTGCTGCGC
101 AGCCACTACC GCGAGGTGCT GCCGCTGGCC ACGTTCGTGC GGCGCCTGGG
151 GCCCCAGGGC TGGCGGCTGG TGCAGCGCGG GGACCCGGCG GCTTTCCGCG
201 CGNTGGTGGC CCANTGCNTG GTGTGCGTGC CCTGGGANGN ANGGCNGCCC
251 CCCGCCGCC CCTCCTTCCG CCAGGTGTCC TGCCTGAANG ANCTGGTGGC
301 CCGAGTGTGC CANANGCTGT GCGANCGCGG CGGAANAAC GTGCTGGCCT
351 TCGGCTTCGC GCTGCTGGAC GGGGCCCGCG GGGGCCCCC CGAGGCCTTC
401 ACCACCAGCG TGCGCAGCTA CCTGCCCAAC ACGGTGACCG ACGCACGTGC
451 GGGGAGCGGG GCGTGGGGGC TGCTGCTGCG CCGCGTGGGC GACGACGTGC
501 TGGTTCACCT GCTGGCACGC TGCGCGNTNT TTGTGCTGGT GGNTCCACAG
551 TGCGCCTACC ANGTTGTGCG GCCGCCGCTG TACCAGCTCG GCGCTGCNAC
601 TCAGGCCCGG CCCCCGCCAC ACGCTANTGG ACCCGAANGC GTCTGGGATC
651 CAACGGCCCT GGAACCATAG CGTCAGGGAG GCCGGGGTCC CCCTGGGCTG
701 CCAGCCCCGG GTGCGAGGAG GCGCGGGGGC AGTGCCAGCC GAAGTCTGCC
751 GTTGCCCAAG AGGCCCAGG GTGGCGCTGC CCTGAGCCG GAGCGGACCG
801 CCGTTGGGCA GGGGTCTTGG GCCACCCCG GCAGGACGCC TGGACCGAGT
851 GACCGTGGTT TCTGTGTGGT GTCACCTGCC AGACCCGCGG AAGAAGCCAC
901 CTCTTTGGAG GGTGCGCTCT CTGGCACGCG CCACTCCAC CCATCCGTGG
951 GCGGCCAGCA CCACGCGGGC CCCCCATCCA CATCGCGGCC ACCACGTCTT
1001 GGGACACGCC TTGTCCCCCG GTGTACGCCG AGACCAAGCA CTTCTCTAC
1051 TCCTCAGGCG ACAAGNACAC TCGNCCCTC CTTCTACTC AATATATCTG
1101 AGGCCAGGCC TGACTGGCGT TCGGGAGGTT CGTGGAGACA NTCTTTCTGG
1151 TTCCAGGCCT TGGATGCCAG GATTCCCCGC AGGTTGCCCC GCCTGCCCCA
1201 GCGNTACTGG CAAATGCGGC CCCTGTTTCT GGAGCTGCTT GGAACACAG
1251 CGCAGTGCCC CTACGGGGTG TTCTCAAGA CGCACTGCCC GCTGCGAGCT
1301 GCGGTACCCC CAGCAGCCGG TGTCTGTGCC CGGGAGAAGC CCCAGGGCTC
1351 TGTGGCGGCC CCCGAGGAGG AGGAACACAG ACCCCCGTCG CCTGGTGACG
1401 CTGCTCCGCC AGCACAGCAG CCCCTGGCAG GTGTACGGCT TCGTGCGGGC
1451 CTGCCCTGCG CGGCTGGTGC CCCCAGGCCT CTGGGGCTCC AGGCACAACG
1501 AACGCCGCTT CCTCAGGAAC ACCAAGAAGT TCATCTCCCT GGGGAAGCAT
1551 GCCAAGCTCT CGCTGCAGGA GCTGACGTGG AAGATGAGCG TGCGGGACTG
1601 CGCTTGCTG CGCAGGAGCC CAGGGGTTGG CTGTGTTCCG GCCCGAGAGC
1651 ACCGTCTGCG TGAGGAGATC CTGGCCAAGT TCCTGCACTG GCTGATGAGT
1701 GTGTACGTCG TCGAGCTGCT CAGGTCTTTC TTTTATGTCA CGGAGACCAC
1751 GTTTCAAAAG AACAGGCTCT TTTTCTACCG GAAGAGTGTC TGGAGCAAGT
1801 TGCAAAGCAT TGGAATCAGA CAGCACTTGA AGAGGGTGCA GCTGCGGGAG
1851 CTGTCGGAAG CAGAGGTCAG GCAGCATCGG GAAGCCAGGC CCGCCCTGCT
1901 GACGTCCAGA CTCCGTTCA TCCCCAAGCC TGACGGGCTG CGGCCGATTG
1951 TGAACATGGA CTACGTCGTG GGAGCCAGAA CGTTCCGCG AGAAAAGAGG
2001 GCCGAGCGTC TCACCTCGAG GGTGAAGGCA CTGTTACGCG TGCTCAACTA
2051 CGAGCGGGCG CGGCGCCCCG GCCTCCTGGG CGCCTCTGTG CTGGGCCTGG
2101 ACGATATCCA CAGGGCCTGG CGCACCTTCG TGCTGCGTGT GCGGGCCCAG
2151 GACCCGCCCG CTGAGCTGTA CTTTGTCAAG GTGGATGTGA CGGGCGCGTA
2201 CGACACCATC CCCCAGGACA GGCTCACGGA GGTCACTGCC AGCATCATCA
2251 AACCCAGAA CACGTACTGC GTGCGTCGGT ATGCCGTGGT CCAGAAGGCC
2301 GCCCATGGGC ACGTCCGCAA GGCCTTCAAG AGCCACGTCT CTACCTTGAC
2351 AGACCTCCAG CCGTACATGC GACAGTTCGT GGCTCACCTG CAGGANAACA
2401 GCCCGCTGAG GGATGCCGTC GTCATCGAGC AGAGCTCCTC CCTGAATGAG
2451 GCCAGCAGTG GCCTCTTCGA CGTCTTCCTA CGTTTCATGT GCCACCACGC

```

FIG. 71

```

2501 CGTGCGCATC AGGGGCAAGT CCTACGTCCA GTGCCAGGGG ATCCCGCAGG
2551 GCTCCATCCT CTCCACGCTG CTCTGCAGCC TGTGCTACGG CGACATGGAG
2601 AACAAAGCTGT TTGCGGGGAT TCGGCGGGAC GGGCTGCTCC TGCCTTTGGT
2651 GGATGATTTC TTGTTGGTGA CACCTCACCT CACCCACGCG AAAACCTTCC
2701 TCAGGACCCT GGTCCGAGGT GTCCCTGAGT ATGGCTGCGT GGTGAACTTG
2751 CGGAAGACAG TGGTGAAC TT CCCTGTAGAA GACGAGGCCC TGGGTGGCAC
2801 GGCCTTTTGTT CAGATGCCGG CCCACGGCCT ATTCCCCTGG TCGGCGCTGC
2851 TGCTGGATAC CCGGACCCTG GAGGTGCAGA GCGACTACTC CAGCTATGCC
2901 CGGACCCTCA TCAGAGCCAG TCTCACCTTC AACCGCGGCT TCAAGGCTGG
2951 GAGGAACATG CGTCGCAAC TCTTTGGGGT CTGCGGCTG AAGTGTCAAC
3001 GCCTGTTTCT GGATTTGCAG GTGAACAGCC TCCAGACGGT GTGCACCAAC
3051 ATCTACAAGA TCCTCCTGCT GCAGGCGTAC AGGTTTCACG CATGTGTGCT
3101 GCAGCTCCCA TTTCATCAGC AAGTTTGGA GAACCCACA TTTTCTCTGC
3151 GCGTCATCTC TGACACGGCC TCCCTCTGCT ACTCCATCCT GAAAGCCAAG
3201 AACGCAGGGA TGTCGCTGGG GGCCAAGGGC GCCGCCGGCC CTCTGCCCTC
3251 CGAGGCCGTG CAGTGGCTGT GCCACCAAGC ATTCCTGCTC AAGCTGACTC
3301 GACACCGTGT CACCTACGTG CCACTCCTGG GGTCACCTAG GACAGCCAGC
3351 ACGCAGCTGA GTCGGAAGCT CCCGGGGACG ACGCTGACTG CCCTGGAGGC
3401 CGCAGCCAAC CCGGCACTGC CCTCAGACTT CAAGACCATC CTGGACTGAT
3451 GGCCACCCGC CCACAGCCAG GCCGAGAGCA GACACCAGCA GCCCTGTCAC
3501 GCCGGGCTCT ACGTCCCAGG GAGGGAGGGG CGGCCCACAC CCAGGCCCGC
3551 ACCGCTGGGA GTCTGAGGCC TGAGTGAGTG TTTGGCCGAG GCCTGCATGT
3601 CCGGCTGAAG GCTGAGTGTC CGGCTGAGGC CTGAGCGAGT GTCCAGCCAA
3651 GGGCTGAGTG TCCAGCACAC CTGCCGTCTT CACTTCCCCA CAGGCTGGCG
3701 CTCGGCTCCA CCCCAGGGCC AGCTTTTCCT CACCAGGAGC CCGGCTTCCA
3751 CTCCCCACAT AGGAATAGTC CATCCCCAGA TTCGCCATTG TTCACCCCTC
3801 GCCCTGCCCT CCTTTGCCTT CCACCCCCAC CATCCAGGTG GAGACCCTGA
3851 GAAGGACCCT GGGAGCTCTG GGAATTTGGA GTGACCAAAG GTGTGCCCTG
3901 TACACAGGCG AGGACCCTGC ACCTGGATGG GGGTCCCTGT GGGTCAAATT
3951 GGGGGGAGGT GCTGTGGGAG TAAAATACTG AATATATGAG TTTTTCAGTT
4001 TTGAAAAAAA AAAAAAAAAA AAAAAAAAAA

```

FIG. 71
(CONTINUED)

GCAGCGCTGCGTCTGCTGCGCACGTGGGAAGCCCTGGCCCCGGCCACCCCCGGATGCC
 1-----+-----+-----+-----+-----+ 60
 CGTCGCGACGCAGGACGACCGCTGCACCCCTTCGGGACCGGGCGGTGGGGCGCTACGG

a A A L R P A A H V G S P G P G H P R D A -
 b Q R C V L L R T W E A L A P A T P A M P -
 c S A A S C C A R G K P W P R P P P R C R -

GC GCGCTCCCCGCTGCGGAGCCGTGCGCTCCCTGCTGCGCAGCCACTACCGCGAGGTGCT
 61-----+-----+-----+-----+-----+ 120
 CGCGCGAGGGCGACGGCTCGGCACGCGAGGGACGACGCGTCGGTGATGGCGCTCCACGA

a A R S P L P S R A L P A A Q P L P R G A -
 b R A P R C R A V R S L L R S H Y R E V L -
 c A L P A A E P C A P C C A A T T A R C C -

GCCGCTGGCCACGTTGCTGCGGCGCTGGGGCCCCAGGGCTGGCGGCTGGTGACGCGCG
 121-----+-----+-----+-----+-----+ 180
 CGCGGACCGGTGCAAGCACGCCGCGGACCCCGGGTCCCGACCGCGACCACTGCGCGC

a A A G H V R A A P G A P G L A A G A A R -
 b P L A T F V R R L G P Q G W R L V Q R G -
 c R W P R S C G A W G P R A G G W C S A G -

GGACCCGCGCGCTTTCGCGCGNTGGTGCCCANITGNTGGTGCGTGCCTGGGANGN
 181-----+-----+-----+-----+-----+ 240
 CCTGGGCCCCCGAAGGCGCGCNACCACCGGGTNACGNACCACACGCACGGGACCCNTCN

a G P G G F P R ? G G P ? ? G V R A L G ? -
 b D P A A F R A ? V A ? C ? V C V P W ? ? -
 c T R R L S A R W W P ? A W C A C P G ? ? -

ANGGCGNCCCCCGCGCCCCCTCCTTCGCGCAGGTGTCTGCGCTGAANGANCTGGTGCG
 241-----+-----+-----+-----+-----+ 300
 TNCCGNCGGGGCGCGCGGGGAGGAAGGCGGTCCACAGGACGGACTTNTNGACCACCG

a ? A A P R R P L L P P G V L P E ? ? G G -
 b ? ? P P A A P S F R Q V S C L ? ? L V A -
 c G ? P P P P P P S A R C P A * ? ? W W P -

CCGAGTGCTGCANANGCTGTGCGANCGCGCGCGAANAACGTGCTGGCCTTCGGCTTCGC
 301-----+-----+-----+-----+-----+ 360
 GGCTCAGACGTTNTNCGACACGCTNGCGCGCGCTTNTTGACACCGGAAGCGGAAGCG

a P S A A ? A V R ? R R E ? R A G L R L R -
 b R V L ? ? L C ? R G A ? N V L A F G F A -
 c E C C ? ? C A ? A A R ? T C W P S A S R -

GCTGCTGGACGGGGCCCGCGGGGGCCCCCGAGGCCTTCACCACCAGCGTGCGCAGCTA
 361-----+-----+-----+-----+-----+ 420
 CGACGAOCTGCCCGGGGCGCCCGGGGGGGCTCCGGAAGTGGTGGTCGCACGCGTCGAT

a A A G R G P R G P P R G L H H Q R A Q L -
 b L L D G A R G G P P E A F T T S V R S Y -
 c C W T G P A G A P P R P S P P A C A A T -

CCTGCCCAACACGCTGACCGACGCACTGCGGGGGAGCGGGCGTGCGGGCTGCTGCTGCG
 421-----+-----+-----+-----+-----+ 480
 GGACGGGTGTGCCACTGGCTGCGTGACGCCCCCTCGCCCCGACCCCGACGACGACGC

a P A Q H G D R R T A G E R G V G A A A A -
 b L P N T V T D A L R G S G A W G L L L R -
 c C P T R * P T H C G G A G R G G C C C A -

FIG. 72

89/103

```

a   P R G R R R A G S P A G T L R ? ? C A G -
b   R V G D D V L V H L L A R C A ? F V L V -
c   A W A T T C W F T C W H A A R ? L C W W -

541  G G N T C C C A G C T G C G C C T A C C A N G T G T G C G G C C G C G C T G T A C C A G C T C G G C G C T G C N A C
      +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
      C C N A G G G T C G A C G C G G A T G G T N C A C A C G C C G G G C G G C A T G G T C G A G C C G C G A C G N T G
      +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
      600

a   G S Q L R L P ? V R A A A V P A R R C ? -
b   ? P S C A Y ? V C G P P L Y Q L G A A T -
c   ? P A A P T ? C A G R R C T S S A L ? L -

601  T C A G G C C G G C C C C C G C C A C A C G C T A N T G G A C C C G A A N G C G T C T G G G A T C C A A C G G G C C T
      +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
      A G T C C G G G C C G G G G G G G T G T G C A T N A C C T G G G C T T N C G C A G A C C C T A G G T T G C C C G G A
      +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
      660

a   S G P A P A T R ? W T R ? R L G S N G P -
b   Q A R P P P H A ? G P E ? V W D P T G L -
c   R P G P R H T L ? D P ? A S G I Q R A W -

661  G G A A C C A T A G C G T C A G G G A G G C C G G G T C C C C C T G G G C T G C C A G C C C G G T G C G A G G A G
      +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
      C C T T G G T A T C G C A G T C C C T C C G G C C C A G G G G A C C C G A C G G T C G G G G C C A C G T C C T C
      +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
      720

a   G T I A S G R P G S P W A A S P G C E E -
b   E P * R Q G G R G P P G L P A P G A R R -
c   N H S V R E A G V P L G C Q P R V R G G -

721  G C G C G G G G C A G T G C C A G C C G A A G T C T G C C G T T G C C C A A G A G G C C C A G G C G T G G C G C T G C
      +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
      C G C G C C C C G T C A C G G T C G G C T C A G A C G G C A A C G G G T T C T C C G G G T C C G C A C C G C G A C G
      +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
      780

a   A R G Q C Q P K S A V A Q E A Q A W R C -
b   R G G S A S R S L P L P K R P R R G A A -
c   A G A V P A E V C R C P R G P G V A L P -

781  C C C T G A G C C G G A G C G G A C G C C C G T T G G G C A G G G T C C T G G G C C C A C C G G G C A G G A C G C C
      +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
      G G G A C T C G G C C T C G C C T G C G G G C A A C C G T C C C C A G G A C C C G G T G G G C C C G T C C T G C G G
      +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
      840

a   P * A G A D A R W A G V L G P P G Q D A -
b   P E P E R T P V G Q G S W A H P G R T P -
c   L S R S G R P L G R G P G P T R A G R L -

841  T G G A C C G A G T G A C C G T G G T T T C T G T G T G T C A C C T G C C A G A C C C G C C A A G A A G C C A C
      +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
      A C C T G G C T C A C T G G C A C C A A G A C A C C A C A G T G G A C G G T C T G G G C G G C T T C T T C G G T G
      +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
      900

a   W T E * P W F L C G V T C Q T R R R S H -
b   G P S D R G F C V V S P A R P A E E A T -
c   D R V T V V S V W C H L P D P P K K P P -

901  C T C T T T G G A G G G T G C G C T C T C T G G C A C G C C A C T C C C A C C C A T C C G T G G G C C G C C A G C A
      +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
      G A G A A C C T C C C A C G C G A G A C C G T G C G C G T G A G G T G G G T A G G C A C C C G G C G G T C G T
      +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
      960

a   L F G G C A L W H A P L P P I R G P P A -
b   S L E G A L S G T R H S H P S V G R Q H -
c   L W R V R S L A R A T P T H P W A A S T -

961  C C A C G C G G G C C C C C A T C C A C A T C G C G G C C A C C A C G T C C T G G G A C A C G C C T T G T C C C C G G
      +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
      G G T G C G C C C G G G G T A G G T G T A G C C C G T G G T G C A G G A C C C T G T C G G A A C A G G G G C
      +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
      1020

```

FIG. 72
(CONTINUED)

90/103

```

a   P R G P P I H I A A T T S W D T P C P P -
b   H A G P P S T S R P P R P G T R L V P R -
c   T R A P H P H R G H H V L G H A L S P G -

1021 GTGTACGCCGAGACCAAGCACTTCTCTACTCTCTCAGGCGACAAGNACACTGCGNCCCTC
-----+-----+-----+-----+-----+ 1080
CACATGCGGCTCTGGTTCGTGAAGGAGATGAGGAGTCCGCTGTTCTNTGTGACGCNCGGAG

a   V Y A E T K H F L Y S S G D K ? T A ? L -
b   C T P R P S T S S T P Q A T ? T L R P S -
c   V R R D Q A L P L L L R R Q ? H C ? P P -

1081 CTCTCTACTCAATATATCTGAGGCCAGCCTGACTGGCGTTCCGGAGGTTCTGTGGAGACA
-----+-----+-----+-----+ 1140
GAAGGATGAGTTATATAGACTCCGGGTCGGACTGACCGCAAGCCCTCCAAGCACCTCTGT

a   L P T Q Y I * G P A * L A F G R F V E T -
b   F L L N I S E A Q P D W R S G G S W R ? -
c   S Y S I Y L R P S L T G V R E V R G D ? -

1141 NTCTTTCTGGTTCAGGCCTTGGATGCCAGGATTCCCGCAGGTTGCCCGCCTGCCCCA
-----+-----+-----+-----+ 1200
NAGAAAGACCAAGGTCCGGAACCTACGGTCTTAAGGGCGTCCAACGGGGCGGACCGGGT

a   ? F L V P G L G C Q D S P Q V A P P A P -
b   S F W F Q A L D A R I P R R L P R L P Q -
c   L S G S R P W M P G F P A G C P A C P S -

1201 GCGNTACTGGCAAATGCGGCCCTGTTCTGGAGCTGCTTGGGAACCAACGCGAGTGCCC
-----+-----+-----+-----+ 1260
CGCNATGACCGTTTACGCCGGGACAAAGACCTCGACGAACCCCTTGGTGCCGCTCACGGG

a   A ? L A N A A P V S G A A W E P R A V P -
b   R Y W Q M R P L F L E L L G N H A Q C P -
c   ? T G K C G P C F W S C L G T T R S A P -

1261 CTACGGGGTGTTCCTCAAGACGCACTGCCCGCTGCGAGCTGCGGTACCCCGAGCAGCCGG
-----+-----+-----+-----+ 1320
GATGCCCCACAAGGAGTTCTGCGTGACGGGCGACGCTCGACGCCAGTGGGGTCTGTCGCCC

a   L R G V P Q D A L P A A S C G H P S S R -
b   Y G V F L K T H C P L R A A V T P A A G -
c   T G C S S R R T A R C E L R S P Q Q P V -

1321 TGTCTGTGCCCGGAGAAGCCCGAGGCTCTGTGGCGGCCCCGAGGAGGAGGAACACAG
-----+-----+-----+-----+ 1380
ACAGACACGGGCCCTCTTCGGGGTCCCGAGACACCGCGGGGCTCTCTCTCTGTGTCTC

a   C L C P G E A P G L C G G P R G G G T Q -
b   V C A R E K P Q G S V A A P E E E E H R -
c   S V P G R S P R A L W R P P R R R N T D -

1381 ACCCCCGTCGCCTGGTGCAGCTGCTCCGCCAGCACAGCAGCCCCCTGGCAGGTGTACGGCT
-----+-----+-----+-----+ 1440
TGGGGGCAGCGGACCACGTCGACGAGGCGGTCTGTCTGTGGGGACCGTCCACATGCCGA

a   T P V A W C S C S A S T A A P G R C T A -
b   P P S P G A A A P P A Q Q P L A G V R L -
c   P R R L V Q L L R Q H S S P W Q V Y G F -

1441 TCGTGCGGGCCTGCCTGCGCGGGCTGGTGCCCCCAGGCCTCTGGGGCTCCAGGCACAACG
-----+-----+-----+-----+ 1500
AGCACGCGCGGACGCGCGGCGGACCAAGGGGTCCCGGAGACCCCGAGTCCCGTGTTCG

```

FIG. 72
(CONTINUED)

91/103

```

a   S C G P A C A G W C P Q A S G A P G T T -
b   R A G L P A P A G A P R P L G L Q A Q R -
c   V R A C L R R L V P P G L W G S R H N E -

AACGCGCTTCCTCAGGAACACCAAGAAGTTCATCTCCCTGGGGAAGCATGCCAAGCTCT
1501 -----+----- 1560
TGCGGCGAAGGAGTCTTGTGGTTCTTCAAGTAGAGGGACCCCTTCGTACGGTTCGAGA

a   N A A S S G T P R S S S P W G S M P S S -
b   T P L P Q E H Q E V H L P G E A C Q A L -
c   R R F L R N T K K F I S L G K H A K L S -

CGCTGCAGGAGCTGACGTGGAAGATGAGCGTGGGGACTGCGCTTGGCTGCGCAGGAGCC
1561 -----+----- 1620
GCGACGTCTCGACTGCACCTTCTACTCGCAGCCCTGACGCGAACCAGCGCTCTCGG

a   R C R S * R G R * A C G T A L G C A G A -
b   A A G A D V E D E R A G L R L A A Q E P -
c   L Q E L T W K M S V R D C A W L R R S P -

CAGGGGTGGCTGTGTCCGGCCGAGAGCACCCTCTGCGTGAGGAGATCCTGGCCAAGT
1621 -----+----- 1680
GTCCCAACCGACACAAGGCCGGCTCTCGTGGCAGACGCACTCTCTAGGACCGTTCA

a   Q G L A V F R P Q S T V C V R R S W P S -
b   R G W L C S G R R A P S A * G D P G Q V -
c   G V G C V P A A E H R L R E E I L A K F -

TCCTGCACTGGCTGATGAGTGTGTACGTGCTCGAGCTGCTCAGGTCTTTCTTTATGTCA
1681 -----+----- 1740
AGGACGTGACCGACTACTCACATGCAGCAGCTCGACGAGTCCAGAAAGAAAATACACT

a   S C T G * * V C T S S S C S G L S F M S -
b   P A L A D E C V R R R A A Q V F L L C H -
c   L H W L M S V Y V V E L L R S F F Y V T -

CGGAGACCACGTTTCAAAGAAGCAGGCTCTTTTCTACCGGAAGAGTGTCTGGAGCAAGT
1741 -----+----- 1800
GCCTCTGGTGCAAAGTTTCTTGTCCGAGAAAAGATGGCCTTCTCACAGACCTCGTTCA

a   R R P R F K R T G S F S T G R V S G A S -
b   G D H V S K E Q A L F L P E E C L E Q V -
c   E T T F Q K N R L F F Y R K S V W S K L -

TGCAAAGCATTGGAAATCAGACAGCACTTGAAGAGGGTGACGCTGCGGGAGCTGTGGAAG
1801 -----+----- 1860
ACGTTTCGTAAACCTTAGTCTGTGCTGAACCTTCTCCACGTGACGCCCCCTCGACAGCCTTC

a   C K A L E S D S T * R G C S C G S C R K -
b   A K H W N Q T A L E E G A A A G A V G S -
c   Q S I G I R Q H L K R V Q L R E L S E A -

CAGAGGTGAGGAGCATCGGGAAGCCAGGCCCGCCCTGCTGACGTCCAGACTCCGCTTCA
1861 -----+----- 1920
GTCTCCAGTCCGTCGTAGCCCTTCGGTCCGGGGGACGACTGCAGGTCTGAGGCGAAGT

a   Q R S G S I G K P G P P C * R P D S A S -
b   R G Q A A S G S Q A R P A D V Q T P L H -
c   E V R Q H R E A R P A L L T S R L R F I -

TCCCCAAGCCTGACGGGCTGCGGCCGATTGTGAACATGGACTACGTCTGGGAGCCAGAA
1921 -----+----- 1980
AGGGGTTCGACTGCCCCGACCGCGCTAACACTTGTACCTGATGCAGCACCCCTCGGTCTT

```

FIG. 72
(CONTINUED)

92/103

```

a   S P S L T G C G R L * T W T T S W E P E -
b   P Q A * R A A A D C E H G L R R G S Q N -
c   P K P D G L R P I V N M D Y V V G A R T -

CGTTCGCGAGAGAAAGAGGGCCGAGCGTCTCACCTCGAGGGTGAAGGCACTGTTTCAGCG
1981 -----+-----+-----+-----+-----+ 2040
GCAAGGCGTCTCTTTTCTCCCGCTCGCAGAGTGGAGCTCCCACTTCCGTGACAAGTCGC

a   R S A E K R G P S V S P R G * R H C S A -
b   V P Q R K E G R A S H L E G E G T V Q R -
c   F R R E K R A E R L T S R V K A L F S V -

TGCTCAACTACGAGCGGGCGCGGGCCCCCGCCTCTGGGCGCCTCTGTGCTGGGCGCTGG
2041 -----+-----+-----+-----+-----+ 2100
ACGAGTTGATGCTCGCCCGCGCGGGGGCGGAGGACCGCGGAGACACGACCGCGGACC

a   C S T T S G R G A P A S W A P L C W A W -
b   A Q L R A G A A P R P P G R L C A G P G -
c   L N Y E R A R R P G L L G A S V L G L D -

ACGATATCCACAGGGCCTGGCGCACCTTCGTGCTGCGTGTGGGGGCCAGGACCGCGCGC
2101 -----+-----+-----+-----+-----+ 2160
TGCTATAGGTGTCCCGGACCGCGTGAAGCACGACGCACACGCCCGGGTCTGGGCGGGC

a   T I S T G P G A P S C C V C G P R T R R -
b   R Y P Q G L A H L R A A C A G P G P A A -
c   D I H R A W R T F V L R V R A Q D P P P -

CTGAGCTGTACTTTGTCAAGGTGGATGTGACGGGGCGGTACGACACCATCCCCAGGACA
2161 -----+-----+-----+-----+-----+ 2220
GACTCGACATGAAACAGTTCCACCTACACTGCCCGCGCATGCTGTGTTAGGGGGTCTGT

a   L S C T L S R W M * R A R T T P S P R T -
b   * A V L C Q G G C D G R V R H H P P G Q -
c   E L Y F V K V D V T G A Y D T I P Q D R -

GGCTCAGGAGGTTCATCGCCAGCATCATCAAACCCAGAACACGTACTGCGTGGCTCGGT
2221 -----+-----+-----+-----+-----+ 2280
CCGAGTGCCTCCAGTAGCGGTGCTAGTAGTTTGGGGTCTTGTGCATGACGCACGCAGCCA

a   G S R R S S P A S S N P R T R T A C V G -
b   A H G G H R Q H H Q T P E H V L R A S V -
c   L T E V I A S I I K P Q N T Y C V R R Y -

ATGCCGTGGTCCAGAAGGCCGCCATGGGCACGTCCGCAAGGCCTTCAAGAGCCACGTCT
2281 -----+-----+-----+-----+-----+ 2340
TACGGCACCAAGGTCTTCCGGCGGGTACCCGTGCAGGCGTTCGGAAGTCTCGGTGCAGA

a   M P W S R R P P M G T S A R P S R A T S -
b   C R G P E G R P W A R P Q G L Q E P R L -
c   A V V Q K A A H G H V R K A F K S H V S -

CTACCTTGACAGACCTCCAGCCGTACATGCGACAGTTCGTGGCTCACCTGCAGGANAACA
2341 -----+-----+-----+-----+-----+ 2400
GATGGAACGTGTCTGGAGGTGGCATGTACGCTGTCAAGCACCGAGTGGACGTCCTTNTGT

a   L P * Q T S S R T C D S S W L T C R ? T -
b   Y L D R P P A V H A T V R G S P A G ? Q -
c   T L T D L Q P Y M R Q F V A H L Q ? N S -

GCCCGCTGAGGGATGCCGTGCTCATCGAGCAGAGCTCCTCCCTGAATGAGGCCAGCAGTG
2401 -----+-----+-----+-----+-----+ 2460
CGGGCGACTCCCTACGGCAGCAGTAGCTCGTCTCGAGGAGGACTTACTCCGGTGTGAC

```

FIG. 72
(CONTINUED)

93/103

```

a   A R * G M P S S S S R A P P * M R P A V -
b   P A E G C R R H R A E L L P E * G Q Q W -
c   P L R D A V V I E Q S S S L N E A S S G -

GCCTCTTCGACGCTCTTCCTACGCTTCATGTGCCACCAACGCGTGCGCATCAGGGGCAAGT
2461 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2520
CGGAGAAGCTGCAGAAGGATGCGAAGTACACGGTGGTGGCGCACGCGTAGTCCCGGTTCA

a   A S S T S S Y A S C A T T P C A S G A S -
b   P L R R L P T L H V P P R R A H Q G Q V -
c   L F D V F L R F M C H H A V R I R G K S -

CCTACGTCCAGTGCCAGGGGATCCCGCAGGGCTCCATCCTCTCCACGCTGCTCTGCAGCC
2521 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2580
GGATGCAGGTCACGGTCCCTTAGGGCGTCCCGAGGTAGGAGAGGTGCGACGAGACGTCGG

a   P T S S A R G S R R A P S S P R C S A A -
b   L R P V P G D P A G L H P L H A A L Q P -
c   Y V Q C Q G I P Q G S I L S T L L C S L -

TGTGCTACGGCGACATGGAGAACAAGCTGTTTGGCGGGGATTGGCGGGGACGGGCTGCTCC
2581 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2640
ACACGATGCCGCTGTACCTCTTGTTCGACAAACGCCCTAAGCGCCCTGCCCCGACGAGG

a   C A T A T W R T S C L R G F G G T G C S -
b   V L R R H G E Q A V C G D S A G R A A P -
c   C Y G D M E N K L F A G I R R D G L L L -

TGGCTTTGGTGGATGATTTCTTGTGTGGTGACACCTCACCTCACCCACGCGAAAACCTTCC
2641 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2700
ACGCAAACCACTACTAAAGAACAACCACTGTGGAGTGGAGTGGGTGCGCTTTTGGAAAGG

a   C V W W M I S C W * H L T S P T R K P S -
b   A F G G * F L V G D T S P H P R E N L P -
c   R L V D D F L L V T P H L T H A K T F L -

TCAGGACCTTGGTCCGAGGTGTCCCTGAGTATGGCTGGGTGGTGAACCTTGGCGAAGACAG
2701 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2760
AGTCTGGGACCAGGCTCCACAGGACTCATACCGACGCCACCTTGAACGCCCTTCTGTGTC

a   S G P W S E V S L S M A A W * T C G R Q -
b   Q D P G P R C P * V W L R G E L A E D S -
c   R T L V R G V P E Y G C V V N L R K T V -

TGGTGAACCTTCCCTGTAGAAGACGAGGCCCTGGGTGGCACGGCTTTTGTTCAGATGCCGG
2761 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2820
ACCACTTGAAGGGACATCTTCTGCTCCGGGACCCACCGTGCCGAAAACAAGTCTACGGCC

a   W * T S L * K T R P W V A R L L F R C R -
b   G E L P C R R R G P G W H G F C S D A G -
c   V N F P V E D E A L G G T A F V Q M P A -

CCCACGGCCTATTCCCCTGGTGGCGCTGCTGCTGGATACCCGACCCCTGGAGGTGCAGA
2821 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2880
GGGTGCCGGATAAGGGGACCACGCCGACGACCTATGGGCCTGGGACCTCCACGTCT

a   P T A Y S P G A A C C W I P G P W R C R -
b   P R P I P L V R P A A G Y P D P G G A E -
c   H G L F P W C G L L L D T R T L E V Q S -

GCGACTACTCCAGCTATGCCCGGACCTCCATCAGAGCCAGTCTCACCTTCAACCGCGGCT
2881 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2940
CGCTGATGAGGTGATACGGGCTGGAGGTAGTCTGGGTGAGAGTGAAGTTGGCGCGCA

```

FIG. 72
(CONTINUED)

94/103

```

a   A T T P A M P G P P S E P V S P S T A A -
b   R L L Q L C P D L H Q S Q S H L Q P R L -
c   D Y S S Y A R T S I R A S L T F N R G F -

TCAAGGCTGGGAGGAACATGCGTCGCAAACTCTTTGGGGTCTTGGGGCTGAAGTGTACACA
2941 -----+-----+-----+-----+-----+ 3000
AGTTCGACCCCTCCCTGTACGCAGCGTTTGAGAAACCCAGAACGCCGACTTCACAGTGT

a   S R L G G T C V A N S L G S C G * S V T -
b   Q G W E E H A S Q T L W G L A A E V S Q -
c   K A G R N M R R K L F G V L R L K C H S -

GCCTGTTTCTGGATTTCAGGTGAACAGCCTCCAGACGGTGTGCACCAACATCTACAAGA
3001 -----+-----+-----+-----+-----+ 3060
CGGACAAAGACCTAAACGTCCTACTTGTGCGAGGTCTGCCACACGTGGTTGTAGATGTTCT

a   A C F W I C R * T A S R R C A P T S T R -
b   P V S G F A G E Q P P D G V H Q H L Q D -
c   L F L D L Q V N S L Q T V C T N I Y K I -

TCCTCCTGCTGCAGGCGTACAGGTTTCACGCATGTGTGCTGCAGCTCCCATTTTCATCAGC
3061 -----+-----+-----+-----+-----+ 3120
AGGAGGACGACGTCCGCATGTCCAAAGTGGTACACACGACGTGAGGGTAAAGTAGTCG

a   S S C C R R T G F T H V C C S S H F I S -
b   P P A A G V Q V S R M C A A A P I S S A -
c   L L L Q A Y R F H A C V L Q L P F H Q Q -

AAGTTTGAAGAACCCACATTTTCTGCGCGTCATCTCTGACACGGCCTCCCTCTGCT
3121 -----+-----+-----+-----+-----+ 3180
TTCAAACCTTCTTGGGGTGTAAAAAGGACCGCGAGTAGAGACTGTGCCGGAGGAGACGA

a   K F G R T P H F S C A S S L T R P P S A -
b   S L E E P H I F P A R H L * H G L P L L -
c   V W K N P T F F L R V I S D T A S L C Y -

ACTCCATCCTGAAAGCCAAGAACGAGGGATGTGCTGGGGGCAAGGCGCGCGCGCGCC
3181 -----+-----+-----+-----+-----+ 3240
TGAGGTAGGACTTTTCGGTTCTTGCGTCCCTACAGCGACCCCCGGTTCCCGCGCGCGCGCG

a   T P S * K P R T Q G C R W G P R A P P A -
b   L H P E S Q E R R D V A G G Q G R R R P -
c   S I L K A K N A G M S L G A K G A A G P -

CTCTGCCCTCCGAGGCGGTGCAGTGGCTGTGCCACCAAGCATTCCTGCTCAAGCTGACTC
3241 -----+-----+-----+-----+-----+ 3300
GAGACGGGAGGCTCCGGCACGTACCGACACGGTGGTTGTAAGGACGAGTTCGACTGAG

a   L C P P R P C S G C A T K H S C S S * L -
b   S A L R G R A V A V P P S I P A Q A D S -
c   L P S E A V Q W L C H Q A F L L K L T R -

GACACCGTGTACCTACGTGCCACTCCTGGGGTCACTCAGGACAGCCAGACGAGCTGA
3301 -----+-----+-----+-----+-----+ 3360
CTGTGGCACAGTGGATGCAGGTGAGGACCCAGTGAGTCTCTGTCGGTCTGCGTCGACT

a   D T V S P T C H S W G H S G Q P R R S * -
b   T P C H L R A T P G V T Q D S P D A A E -
c   H R V T Y V P L L G S L R T A Q T Q L S -

GTCCGAAGCTCCCGGGGACGACGTGACTGCCCTGGAGGCCGAGCCAAACCCGCACTGC
3361 -----+-----+-----+-----+-----+ 3420
CAGCCTTCGAGGGCCCTGCTGCGACTGACGGGACCTCCGGCGTCCGTTGGGCGGTGACG

```

FIG. 72
(CONTINUED)

FIG. 72
(CONTINUED)

96/103

```
a   E T L R R T L G A L G I W S D Q R C A L -
b   R P * E G P W E L W E F G V T K G V P C -
c   D P E K D P G S S G N L E * P K V C P V -

TACACAGGCGAGGACCCCTGCACCTGGATGGGGGTCCCTGTGGGTCAAATTGGGGGGAGGT
3901 -----+-----+-----+-----+-----+ 3960
ATGTGTCCGCTCCTGGGACGTGGACCTACCCCCAGGGACACCCAGTTTAAACCCCTCCA

a   Y T G E D P A P G W G S L W V K L G G G -
b   T Q A R T L H L D G G P C G S N W G E V -
c   H R R G P C T W M G V P V G Q I G G R C -

GCTGTGGGAGTAAAATACTGAATATATGAGTTTTCAGTTTGTAAAAA
3961 -----+-----+-----+-----+-----+ 4020
CGACACCCCTCATTTTATGACTTATATACTCAAAAAGTCAAACTTTT

a   A V G V K Y * I Y E F F S F E K K K K K -
b   L W E * N T E Y M S F S V L K K K K K K -
c   C G S K I L N I * V F Q F * K K K K K K -

AAAAA
4021 ----- 4029
TTTTTTTTT

a   K K K -
b   K K -
c   K K -
```

FIG. 72
(CONTINUED)

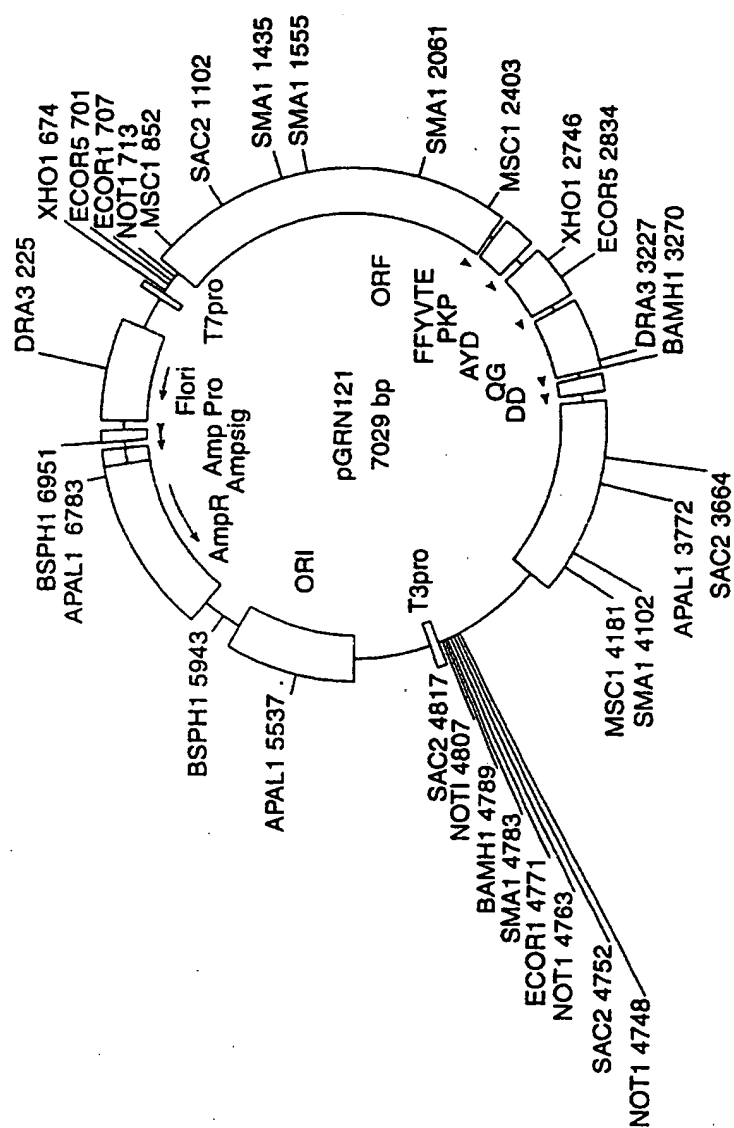


FIG. 73

98/103

```

                                1
                                met
GCAGCGCTGCGTCCTGCTGCGCACGTGGGAAGCCCTGGCCCCGGCCACCCCCGCG ATG

                                10
pro arg ala pro arg cys arg ala val arg ser leu leu arg ser
CCG CGC GCT CCC CGC TGC CGA GCC GTG CGC TCC CTG CTG CGC AGC

                                20
his tyr arg glu val leu pro leu ala thr phe val arg arg leu
CAC TAC CGC GAG GTG CTG CCG CTG GCC ACG TTC GTG CGG CGC CTG

                                30
gly pro gln gly trp arg leu val gln arg gly asp pro ala ala
GGG CCC CAG GGC TGG CGG CTG GTG CAG CGC GGG GAC CCG GCG GCT

                                40
phe arg ala leu val ala gln cys leu val cys val pro trp asp
TTC CGC GCG CTG GTG GCC CAG TGC CTG GTG TGC GTG CCC TGG GAC

                                50
ala arg pro pro pro ala ala pro ser phe arg gln val ser cys
GCA CGG CCG CCC CCC GCC GCC CCC TCC TTC CGC CAG GTG TCC TGC

                                60
leu lys glu leu val ala arg val leu gln arg leu cys glu arg
CTG AAG GAG CTG GTG GCC CGA GTG CTG CAG AGG CTG TGC GAG CGC

                                70
gly ala lys asn val leu ala phe gly phe ala leu leu asp gly
GGC GCG AAG AAC GTG CTG GCC TTC GGC TTC GCG CTG CTG GAC GGG

                                80
ala arg gly gly pro pro glu ala phe thr thr ser val arg ser
GCC CGC GGG GGC CCC CCC GAG GCC TTC ACC ACC AGC GTG CGC AGC

                                90
tyr leu pro asn thr val thr asp ala leu arg gly ser gly ala
TAC CTG CCC AAC ACG GTG ACC GAC GCA CTG CGG GGG AGC GGG GCG

                                100
trp gly leu leu leu arg arg val gly asp asp val leu val his
TGG GGG CTG CTG CTG CGC CGC GTG GGC GAC GAC GTG CTG GTT CAC

                                110
leu leu ala arg cys ala leu phe val leu val ala pro ser cys
CTG CTG GCA CGC TGC GCG CTC TTT GTG CTG GTG GCT CCC AGC TGC

                                120
ala tyr gln val cys gly pro pro leu tyr gln leu gly ala ala
GCC TAC CAG GTG TGC GGG CCG CCG CTG TAC CAG CTC GGC GCT GCC

                                130
thr gln ala arg pro pro pro his ala ser gly pro arg arg arg
ACT CAG GCC CGG CCC CCG CCA CAC GCT AGT GGA CCC CGA AGG CGT

```

FIG. 74

99/103

| | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
| 200 | | | | | | | | | | | | 210 | | | |
| leu | gly | cys | glu | arg | ala | trp | asn | his | ser | val | arg | glu | ala | gly | |
| CTG | GGA | TGC | GAA | CGG | GCC | TGG | AAC | CAT | AGC | GTC | AGG | GAG | GCC | GGG | |
| 220 | | | | | | | | | | | | | | | |
| val | pro | leu | gly | leu | pro | ala | pro | gly | ala | arg | arg | arg | gly | gly | |
| GTC | CCC | CTG | GGC | CTG | CCA | GCC | CCG | GGT | GCG | AGG | AGG | CGC | GGG | GGC | |
| 230 | | | | | | | | | | | | 240 | | | |
| ser | ala | ser | arg | ser | leu | pro | leu | pro | lys | arg | pro | arg | arg | gly | |
| AGT | GCC | AGC | CGA | AGT | CTG | CCG | TTG | CCC | AAG | AGG | CCC | AGG | CGT | GGC | |
| 250 | | | | | | | | | | | | | | | |
| ala | ala | pro | glu | pro | glu | arg | thr | pro | val | gly | gln | gly | ser | trp | |
| GCT | GCC | CCT | GAG | CCG | GAG | CGG | ACG | CCC | GTT | GGG | CAG | GGG | TCC | TGG | |
| 260 | | | | | | | | | | | | 270 | | | |
| ala | his | pro | gly | arg | thr | arg | gly | pro | ser | asp | arg | gly | phe | cys | |
| GCC | CAC | CCG | GGC | AGG | ACG | CGT | GGA | CCG | AGT | GAC | CGT | GGT | TTC | TGT | |
| 280 | | | | | | | | | | | | | | | |
| val | val | ser | pro | ala | arg | pro | ala | glu | glu | ala | thr | ser | leu | glu | |
| GTG | GTG | TCA | CCT | GCC | AGA | CCC | GCC | GAA | GAA | GCC | ACC | TCT | TTG | GAG | |
| 290 | | | | | | | | | | | | 300 | | | |
| gly | ala | leu | ser | gly | thr | arg | his | ser | his | pro | ser | val | gly | arg | |
| GGT | GCG | CTC | TCT | GGC | ACG | CGC | CAC | TCC | CAC | CCA | TCC | GTG | GGC | CGC | |
| 310 | | | | | | | | | | | | | | | |
| gln | his | his | ala | gly | pro | pro | ser | thr | ser | arg | pro | pro | arg | pro | |
| CAG | CAC | CAC | GCG | GGC | CCC | CCA | TCC | ACA | TCG | CGG | CCA | CCA | CGT | CCC | |
| 320 | | | | | | | | | | | | 330 | | | |
| trp | asp | thr | pro | cys | pro | pro | val | tyr | ala | glu | thr | lys | his | phe | |
| TGG | GAC | ACG | CCT | TGT | CCC | CCG | GTG | TAC | GCC | GAG | ACC | AAG | CAC | TTC | |
| 340 | | | | | | | | | | | | | | | |
| leu | tyr | ser | ser | gly | asp | lys | glu | gln | leu | arg | pro | ser | phe | leu | |
| CTC | TAC | TCC | TCA | GGC | GAC | AAG | GAG | CAG | CTG | CGG | CCC | TCC | TTC | CTA | |
| 350 | | | | | | | | | | | | 360 | | | |
| leu | ser | ser | leu | arg | pro | ser | leu | thr | gly | ala | arg | arg | leu | val | |
| CTC | AGC | TCT | CTG | AGG | CCC | AGC | CTG | ACT | GGC | GCT | CGG | AGG | CTC | GTG | |
| 370 | | | | | | | | | | | | | | | |
| glu | thr | ile | phe | leu | gly | ser | arg | pro | trp | met | pro | gly | thr | pro | |
| GAG | ACC | ATC | TTT | CTG | GGT | TCC | AGG | CCC | TGG | ATG | CCA | GGG | ACT | CCC | |
| 380 | | | | | | | | | | | | 390 | | | |
| arg | arg | leu | pro | arg | leu | pro | gln | arg | tyr | trp | gln | met | arg | pro | |
| CGC | AGG | TTG | CCC | CGC | CTG | CCC | CAG | CGC | TAC | TGG | CAA | ATG | CGG | CCC | |
| 400 | | | | | | | | | | | | | | | |
| leu | phe | leu | glu | leu | leu | gly | asn | his | ala | gln | cys | pro | tyr | gly | |
| CTG | TTT | CTG | GAG | CTG | CTT | GGG | AAC | CAC | GCG | CAG | TGC | CCC | TAC | GGG | |
| 410 | | | | | | | | | | | | 420 | | | |
| val | leu | leu | lys | thr | his | cys | pro | leu | arg | ala | ala | val | thr | pro | |
| GTG | CTC | CTC | AAG | ACG | CAC | TGC | CCG | CTG | CGA | GCT | GCG | GTC | ACC | CCA | |

FIG. 74
(CONTINUED)

100/103

430
ala ala gly val cys ala arg glu lys pro gln gly ser val ala
GCA GCC GGT GTC TGT GCC CGG GAG AAG CCC CAG GGC TCT GTG GCG

440 450
ala pro glu glu glu asp thr asp pro arg arg leu val gln leu
GCC CCC GAG GAG GAG GAC ACA GAC CCC CGT CGC CTG GTG CAG CTG

460
leu arg gln his ser ser pro trp gln val tyr gly phe val arg
CTC CGC CAG CAC AGC AGC CCC TGG CAG GTG TAC GGC TTC GTG CGG

470 480
ala cys leu arg arg leu val pro pro gly leu trp gly ser arg
GCC TGC CTG CGC CGG CTG GTG CCC CCA GGC CTC TGG GGC TCC AGG

490
his asn glu arg arg phe leu arg asn thr lys lys phe ile ser
CAC AAC GAA CGC CGC TTC CTC AGG AAC ACC AAG AAG TTC ATC TCC

500 510
leu gly lys his ala lys leu ser leu gln glu leu thr trp lys
CTG GGG AAG CAT GCC AAG CTC TCG CTG CAG GAG CTG ACG TGG AAG

520
met ser val arg asp cys ala trp leu arg arg ser pro gly val
ATG AGC GTG CGG GAC TGC GCT TGG CTG CGC AGG AGC CCA GGG GTT

530 540
gly cys val pro ala ala glu his arg leu arg glu glu ile leu
GGC TGT GTT CCG GCC GCA GAG CAC CGT CTG CGT GAG GAG ATC CTG

550
ala lys phe leu his trp leu met ser val tyr val val glu leu
GCC AAG TTC CTG CAC TGG CTG ATG AGT GTG TAC GTC GTC GAG CTG

560 570
leu arg ser phe phe tyr val thr glu thr thr phe gln lys asn
CTC AGG TCT TTC TTT TAT GTC ACG GAG ACC ACG TTT CAA AAG AAC

580
arg leu phe phe tyr arg pro ser val trp ser lys leu gln ser
AGG CTC TTT TTC TAC CGG CCG AGT GTC TGG AGC AAG TTG CAA AGC

590 600
ile gly ile arg gln his leu lys arg val gln leu arg glu leu
ATT GGA ATC AGA CAG CAC TTG AAG AGG GTG CAG CTG CGG GAG CTG

610
ser glu ala glu val arg gln his arg glu ala arg pro ala leu
TCG GAA GCA GAG GTC AGG CAG CAT CGG GAA GCC AGG CCC GCC CTG

620 630
leu thr ser arg leu arg phe ile pro lys pro asp gly leu arg
CTG ACG TCC AGA CTC CGC TTC ATC CCC AAG CCT GAC GGG CTG CGG

640
pro ile val asn met asp tyr val val gly ala arg thr phe arg
CCG ATT GTG AAC ATG GAC TAC GTC GTG GGA GCC AGA ACG TTC CGC

FIG. 74
(CONTINUED)

101/103

650 arg glu lys arg ala glu arg leu thr ser arg val lys ala leu
 AGA GAA AAG AGG GCC GAG CGT CTC ACC TCG AGG GTG AAG GCA CTG

660
 phe ser val leu asn tyr glu arg ala arg arg pro gly leu leu
 TTC AGC GTG CTC AAC TAC GAG CGG GCG CGG CGC CCC GGC CTC CTG

670
 gly ala ser val leu gly leu asp asp ile his arg ala trp arg
 GGC GCC TCT GTG CTG GGC CTG GAC GAT ATC CAC AGG GCC TGG CGC

680
 thr phe val leu arg val arg ala gln asp pro pro pro glu leu
 ACC TTC GTG CTG CGT GTG CGG GCC CAG GAC CCG CCG CCT GAG CTG

690
 tyr phe val lys val asp val thr gly ala tyr asp thr ile pro
 TAC TTT GTC AAG GTG GAT GTG ACG GGC GCG TAC GAC ACC ATC CCC

700
 gln asp arg leu thr glu val ile ala ser ile ile lys pro gln
 CAG GAC AGG CTC ACG GAG GTC ATC GCC AGC ATC ATC AAA CCC CAG

710
 asn thr tyr cys val arg arg tyr ala val val gln lys ala ala
 AAC ACG TAC TGC GTG CGT CGG TAT GCC GTG GTC CAG AAG GCC GCC

720
 his gly his val arg lys ala phe lys ser his val ser thr leu
 CAT GGG CAC GTC CGC AAG GCC TTC AAG AGC CAC GTC TCT ACC TTG

730
 thr asp leu gln pro tyr met arg gln phe val ala his leu gln
 ACA GAC CTC CAG CCG TAC ATG CGA CAG TTC GTG GCT CAC CTG CAG

740
 glu thr ser pro leu arg asp ala val val ile glu gln ser ser
 GAG ACC AGC CCG CTG AGG GAT GCC GTC GTC ATC GAG CAG AGC TCC

750
 ser leu asn glu ala ser ser gly leu phe asp val phe leu arg
 TCC CTG AAT GAG GCC AGC AGT GGC CTC TTC GAC GTC TTC CTA CGC

760
 phe met cys his his ala val arg ile arg gly lys ser tyr val
 TTC ATG TGC CAC CAC GCC GTG CGC ATC AGG GGC AAG TCC TAC GTC

770
 gln cys gln gly ile pro gln gly ser ile leu ser thr leu leu
 CAG TGC CAG GGG ATC CCG CAG GGC TCC ATC CTC TCC ACG CTG CTC

780
 cys ser leu cys tyr gly asp met glu asn lys leu phe ala gly
 TGC AGC CTG TGC TAC GGC GAC ATG GAG AAC AAG CTG TTT GCG GGG

790
 ile arg arg asp gly leu leu leu arg leu val asp asp phe leu
 ATT CGG CGG GAC GGG CTG CTC CTG CGT TTG GTG GAT GAT TTC TTG

800
 810
 820
 830
 840
 850
 860
 870

FIG. 74
(CONTINUED)

102/103

880
 leu val thr pro his leu thr his ala lys thr phe leu arg thr
 TTG GTG ACA CCT CAC CTC ACC CAC GCG AAA ACC TTC CTC AGG ACC

890
 leu val arg gly val pro glu tyr gly cys val val asn leu arg
 CTG GTC CGA GGT GTC CCT GAG TAT GGC TGC GTG GTG AAC TTG CGG

910
 lys thr val val asn phe pro val glu asp glu ala leu gly gly
 AAG ACA GTG GTG AAC TTC CCT GTA GAA GAC GAG GCC CTG GGT GGC

920
 thr ala phe val gln met pro ala his gly leu phe pro trp cys
 ACG GCT TTT GTT CAG ATG CCG GCC CAC GGC CTA TTC CCC TGG TGC

940
 gly leu leu leu asp thr arg thr leu glu val gln ser asp tyr
 GGC CTG CTG CTG GAT ACC CGG ACC CTG GAG GTG CAG AGC GAC TAC

950
 ser ser tyr ala arg thr ser ile arg ala ser val thr phe asn
 TCC AGC TAT GCC CGG ACC TCC ATC AGA GCC AGT GTC ACC TTC AAC

960
 arg gly phe lys ala gly arg asn met arg arg lys leu phe gly
 CGC GGC TTC AAG GCT GGG AGG AAC ATG CGT CGC AAA CTC TTT GGG

970
 val leu arg leu lys cys his ser leu phe leu asp leu gln val
 GTC TTG CGG CTG AAG TGT CAC AGC CTG TTT CTG GAT TTG CAG GTG

980
 asn ser leu gln thr val cys thr asn ile tyr lys ile leu leu
 AAC AGC CTC CAG ACG GTG TGC ACC AAC ATC TAC AAG ATC CTC CTG

990
 leu gln ala tyr arg phe his ala cys val leu gln leu pro phe
 CTG CAG GCG TAC AGG TTT CAC GCA TGT GTG CTG CAG CTC CCA TTT

1000
 his gln gln val trp lys asn pro thr phe phe leu arg val ile
 CAT CAG CAA GTT TGG AAG AAC CCC ACA TTT TTC CTG CGC GTC ATC

1010
 ser asp thr ala ser leu cys tyr ser ile leu lys ala lys asn
 TCT GAC ACG GCC TCC CTC TGC TAC TCC ATC CTG AAA GCC AAG AAC

1020
 ala gly met ser leu gly ala lys gly ala ala gly pro leu pro
 GCA GGG ATG TCG CTG GGG GCC AAG GGC GCC GCC GGC CCT CTG CCC

1030
 ser glu ala val gln trp leu cys his gln ala phe leu leu lys
 TCC GAG GCC GTG CAG TGG CTG TGC CAC CAA GCA TTC CTG CTC AAG

1040
 leu thr arg his arg val thr tyr val pro leu leu gly ser leu
 CTG ACT CGA CAC CGT GTC ACC TAC GTG CCA CTC CTG GGC TCA CTC

FIG. 74
 (CONTINUED)

103/103

```

      1100                                1110
arg thr ala gln thr gln leu ser arg lys leu pro gly thr thr
AGG ACA GCC CAG ACG CAG CTG AGT CGG AAG CTC CCG GGG ACG ACG

                                1120
leu thr ala leu glu ala ala ala asn pro ala leu pro ser asp
CTG ACT GCC CTG GAG GCC GCA GCC AAC CCG GCA CTG CCC TCA GAC

      1130      1132
phe lys thr ile leu asp OP
TTC AAG ACC ATC CTG GAC TGA TGGCCACCCGCCACAGCCAGGCCGAGAGCAGA

CACCAGCAGCCCTGTACGCGGGCTCTACGTCCCAGGGAGGGAGGGGCGGCCACACCC
AGGCCCCGACCGCTGGGAGTCTGAGGCCTGAGTGAGTGTGTTGGCCGAGGCCTGCATGTCC
GGCTGAAGGCTGAGTGTCCGGCTGAGGCCTGAGCGAGTGTCCAGCCAAGGGCTGAGTGTC
CAGCACACCTGCCGTCTTCACTTCCCCACAGGCTGGCGCTCGGCTCCACCCAGGGCCAG
CTTTTCYTCACCAGGAGCCCGCTTCCACTCCCCACATAGGAATAGTCCATCCCCAGATT
CGCCATTGTTCACCCYTCGCCCTGCCYTCCTTTGCCTTCCACCCCAACCATCCAGGTGGA
GACCCTGAGAAGGACCCTGGGAGCTCTGGGAATTTGGAGTGACCAAAGGTGTGCCCTGTA
CACAGGCGAGGACCCTGCACCTGGATGGGGTCCCTGTGGGTCAAATTGGGGGGAGGTGC
TGTGGGAGTAAAATACTGAATATATGAGTTTTTCAGTTTGRAAAAAAAAAAAAAAAAAAA
AAAAAAAAAA

```

FIG. 74
(CONTINUED)

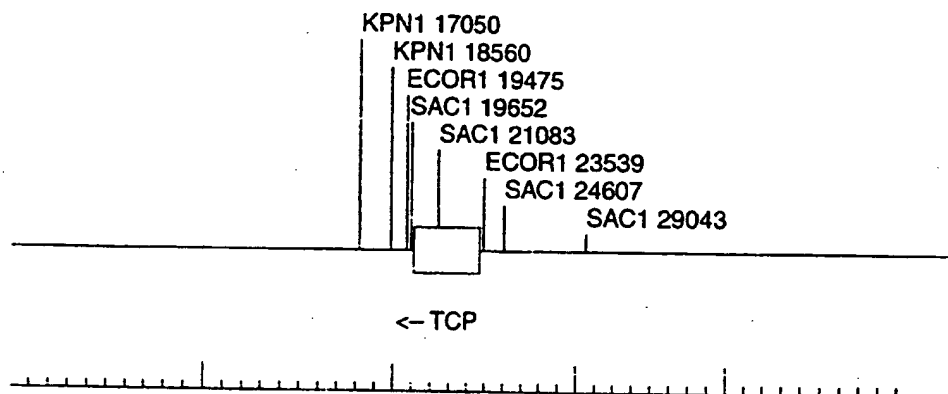


FIG. 75

HUMAN TELOMERASE CATALYTIC SUBUNIT

FIELD OF THE INVENTION

The present invention is related to novel nucleic acids encoding the catalytic subunit of telomerase and related polypeptides. In particular, the present invention is directed to the catalytic subunit of human telomerase. The invention provides methods and compositions relating to medicine, molecular biology, chemistry, pharmacology, and medical diagnostic and prognostic technology.

BACKGROUND OF THE INVENTION

The following discussion is intended to introduce the field of the present invention to the reader.

It has long been recognized that complete replication of the ends of eukaryotic chromosomes requires specialized cell components (Watson, 1972, *Nature New Biol.*, 239:197; Olovnikov, 1973, *J. Theor. Biol.*, 41:181). Replication of a linear DNA strand by conventional DNA polymerases requires an RNA primer, and can proceed only 5' to 3'. When the RNA bound at the extreme 5' ends of eukaryotic chromosomal DNA strands is removed, a gap is introduced, leading to a progressive shortening of daughter strands with each round of replication. This shortening of *telomeres*, the protein-DNA structures physically located on the ends of chromosomes, is thought to account for the phenomenon of cellular senescence or aging (see, e.g., Goldstein, 1990, *Science* 249:1129; Martin et al., 1979, *Lab. Invest.* 23:86; Goldstein et al., 1969, *Proc. Natl. Acad. Sci. USA* 64:155; and Schneider and Mitsui, 1976, *Proc. Natl. Acad. Sci. USA*, 73:3584) of normal human somatic cells *in vitro* and *in vivo*.

The length and integrity of telomeres is thus related to entry of a cell into a senescent stage (i.e., loss of proliferative capacity). Moreover, the ability of a cell to maintain (or increase) telomere length may allow a cell to escape senescence, i.e., to become immortal.

The structure of telomeres and telomeric DNA has been investigated in numerous systems (see, e.g. Harley and Villeponteau, 1995, *Curr. Opin. Genet. Dev.* 5:249). In most organisms, telomeric DNA consists of a tandem array of very simple sequences; in humans and other vertebrates telomeric DNA consists of
5 hundreds to thousands of tandem repeats of the sequence TTAGGG. Methods for determining and modulating telomere length in cells are described in PCT Publications WO 93/23572 and WO 96/41016.

The maintenance of telomeres is a function of a telomere-specific DNA polymerase known as *telomerase*. Telomerase is a ribonucleoprotein (RNP) that uses
10 a portion of its RNA moiety as a template for telomere repeat DNA synthesis (Morin, 1997, *Eur. J. Cancer* 33:750; Yu et al., 1990, *Nature* 344:126; Singer and Gottschling, 1994, *Science* 266:404; Autexier and Greider, 1994, *Genes Develop.*, 8:563; Gilley et al., 1995, *Genes Develop.*, 9:2214; McEachern and Blackburn, 1995, *Nature* 367:403; Blackburn, 1992, *Ann. Rev. Biochem.*, 61:113; Greider,
15 1996, *Ann. Rev. Biochem.*, 65:337). The RNA components of human and other telomerases have been cloned and characterized (see, PCT Publication WO 96/01835 and Feng et al., 1995, *Science* 269:1236). However, the characterization of the protein components of telomerase has been difficult. In part, this is because it has proved difficult to purify the telomerase RNP, which is present in extremely low
20 levels in cells in which it is expressed. For example, it has been estimated that human cells known to express high levels of telomerase activity may have only about one hundred molecules of the enzyme per cell.

Consistent with the relationship of telomeres and telomerase to the proliferative capacity of a cell (i.e., the ability of the cell to divide indefinitely),
25 telomerase activity is detected in immortal cell lines and an extraordinarily diverse set of tumor tissues, but is not detected (i.e., was absent or below the assay threshold) in normal somatic cell cultures or normal tissues adjacent to a tumor (see, U.S. Patent Nos. 5,629,154; 5,489,508; 5,648,215; and 5,639,613; see also, Morin, 1989, *Cell* 59: 521; Shay and Bacchetti 1997, *Eur. J. Cancer* 33:787; Kim et al., 1994, *Science*
30 266:2011; Counter et al., 1992, *EMBO J.* 11:1921; Counter et al., 1994, *Proc. Natl. Acad. Sci. U.S.A.* 91, 2900; Counter et al., 1994, *J. Virol.* 68:3410). Moreover, a

correlation between the level of telomerase activity in a tumor and the likely clinical outcome of the patient has been reported (e.g., U.S. Patent No. 5,639,613, *supra*; Langford et al., 1997, *Hum. Pathol.* 28:416). Telomerase activity has also been detected in human germ cells, proliferating stem or progenitor cells, and activated lymphocytes. In somatic stem or progenitor cells, and in activated lymphocytes, telomerase activity is typically either very low or only transiently expressed (see, Chiu et al., 1996, *Stem Cells* 14:239; Bodnar et al., 1996, *Exp. Cell Res.* 228:58; Taylor et al., 1996, *J. Invest. Dermatology* 106: 759).

Human telomerase is an ideal target for diagnosing and treating human diseases relating to cellular proliferation and senescence, such as cancer. Methods for diagnosing and treating cancer and other telomerase-related diseases in humans are described in U.S. Patent Nos. 5,489,508, 5,639,613, and 5,645,986. Methods for predicting tumor progression by monitoring telomerase are described in U.S. Patent No. 5,639,613. The discovery and characterization of the catalytic protein subunit of human telomerase would provide additional useful assays for telomerase and for disease diagnosis and therapy. Moreover, cloning and determination of the primary sequence of the catalytic protein subunit would allow more effective therapies for human cancers and other diseases related to cell proliferative capacity and senescence.

BRIEF SUMMARY OF THE INVENTION

The present invention provides an isolated, substantially pure, or recombinant protein preparation of a telomerase reverse transcriptase protein, or a variant thereof, or a fragment thereof. In one embodiment the protein is characterized as having a defined motif that has an amino acid sequence:

Trp-R₁-X₇-R₁-R₁-R₂-X-Phe-Phe-Tyr-X-Thr-Glu-X_{8,9}-R₃-R₃-Arg-R₄-X₂-Trp
where X is any amino acid and a subscript refers to the number of consecutive residues. R₁ is leucine or isoleucine. R₂ is glutamine or arginine. R₃ is phenylalanine or tyrosine. and R₄ is lysine or histidine. In one embodiment the protein has a sequence of human TRT. In other embodiments, the invention relates to peptides and polypeptides sharing substantial sequence identity with a subsequence of such proteins.

In a related embodiment the invention provides an isolated, substantially

pure or recombinant nucleic acid that encodes a telomerase reverse transcriptase protein. In one embodiment the nucleic acid encodes a protein comprising an amino acid sequence:

Trp-R₁-X₇-R₁-R₂-X-Phe-Phe-Tyr-X-Thr-Glu-X_{8,9}-R₃-R₃-Arg-R₄-X₂-Trp. In

5 another embodiment, the nucleic acid has a sequence that encodes the human TRT protein. In other embodiments, the invention relates to oligonucleotides and polynucleotides sharing substantial sequence identity or complementarity with a subsequence of such nucleic acids.

In one embodiment, the invention relates to human telomerase reverse transcriptase (hTRT) protein. Thus, in one embodiment, the invention provides an
10 isolated, substantially pure, or recombinant protein preparation of an hTRT protein, or a variant thereof, or a fragment thereof. In one embodiment, the protein is characterized by having an amino acid sequence with at least about 75% or at least about 80% sequence identity to the hTRT protein of Figure 17 (SEQUENCE ID NO: 2), or a
15 variant thereof, or a fragment thereof. In a related aspect, the hTRT protein has the sequence of SEQUENCE ID NO: 2. In some embodiments, the protein has one or more telomerase activities, such as catalytic activity. In one embodiment, the hTRT protein fragment has at least 6 amino acid residues.

The invention also provides a composition comprising an hTRT protein
20 and an RNA. The RNA may be a telomerase RNA, such as a human telomerase RNA. In one embodiment, the hTRT protein and the human telomerase RNA (hTR) from a ribonucleoprotein complex with a telomerase activity.

In one embodiment, the invention provides isolated human telomerase comprising hTRT protein, such as a substantially pure human telomerase comprising
25 hTRT protein and comprising hTR. In one embodiment, the telomerase is at least about 95% pure. The telomerase may be isolated from a cell, such as a recombinant host cell in or a cell that expresses telomerase activity.

In another aspect, the invention provides an isolated, synthetic, substantially pure, or recombinant polynucleotide comprising a nucleic acid sequence
30 that encodes an hTRT protein. In one embodiment, the polynucleotide has a nucleotide sequence encoding an hTRT protein that has an amino acid sequence as set forth in

Figure 17 (SEQUENCE ID NO:2) or a sequence that comprises one or more conservative amino acid (or codon) substitutions or one or more activity-altering amino acid (or codon) substitutions in said amino acid sequence. In a related aspect, the polynucleotide hybridizes under stringent conditions to a polynucleotide having the sequence as set forth in Figure 16 (SEQUENCE ID NO:1). In another related aspect, the nucleotide sequence of the polynucleotide has a smallest sum probability of less than about 0.5 when compared to a nucleotide sequence as set forth in Figure 16 (SEQUENCE ID NO:1) using BLAST algorithm with default parameters.

In another aspect, the invention provides a polynucleotide having a promoter sequence operably linked to the sequence encoding the hTERT protein. The promoter may be a promoter other than the naturally occurring hTERT promoter. In a related aspect, the invention provides an expression vector comprising the promoter of the hTERT.

The invention also provides an isolated, synthetic, substantially pure, or recombinant polynucleotide that is at least ten nucleotides in length and comprises a contiguous sequence of at least ten nucleotides that is identical or exactly complementary to a contiguous sequence in a naturally occurring hTERT gene or hTERT mRNA. In some embodiments the polynucleotide is an RNA, a DNA, or contains one or more non-naturally occurring, synthetic nucleotides. In one aspect, the polynucleotide is identical or exactly complementary to the contiguous sequence of at least ten contiguous nucleotides in a naturally occurring hTERT gene or hTERT mRNA. For example, the polynucleotide may be an antisense polynucleotide. In one embodiment, the antisense polynucleotide comprises at least about 20 nucleotides.

The invention further provides a method of preparing recombinant telomerase by contacting a recombinant hTERT protein with a telomerase RNA component under conditions such that said recombinant protein and said telomerase RNA component associate to form a telomerase enzyme capable of catalyzing the addition of nucleotides to a telomerase substrate. In one embodiment, the hTERT protein has a sequence as set forth in Figure 17 (SEQUENCE ID NO:2). The hTERT protein may be produced in an *in vitro* expression system and mixed with a telomerase RNA or, in another embodiment, the telomerase RNA can be co-expressed in the *in vitro*

expression system. In one embodiment the telomerase RNA is hTR. In an alternative embodiment, the contacting occurs in a cell, such as a human cell. In one embodiment, the cell does not have telomerase activity prior to the contacting of the hTRT and the RNA, or the introduction, such as by transfection, of an hTRT polynucleotide. In one
5 embodiment, the telomerase RNA is expressed naturally by said cell.

The invention also provides a cell, such as a human, mouse, or yeast cell, containing the recombinant polynucleotides of the invention such as a polynucleotide with an hTRT protein coding sequence operably linked a promoter. In particular aspects, the cell is a vertebrate cell, such as a cell from a mammal, for
10 example a human, and has an increased proliferative capacity relative to a cell that is otherwise identical but does not comprise the recombinant polynucleotide or has an increased telomerase activity level relative to a cell that is otherwise identical but does not comprise the recombinant polynucleotide. In some embodiments the cell is immortal.

15 In related embodiments, the invention provides organisms and cells comprising a polynucleotide encoding a human telomerase reverse transcriptase polypeptide, such as a transgenic non-human organism such as a yeast, plant, bacterium, or a non-human animal, for example, a mouse. The invention also provides for transgenic animals and cells from which an hTRT gene has been deleted (knocked-
20 out) or mutated such that the gene does not express a naturally occurring hTRT gene product. Thus, in alternative embodiments, the transgenic non-human animal has a mutated telomerase gene, is an animal deficient in a telomerase activity, is an animal whose TRT deficiency is a result of a mutated gene encoding a TRT having a reduced level of a telomerase activity compared to a wild-type TRT and is an animal having a
25 mutated TRT gene with one or more mutations, including missense mutations, nonsense mutations, insertions, or deletions.

The invention also provides an isolated or recombinant antibody, or fragment thereof, that specifically binds to an hTRT protein. In one embodiment, the antibody binds with an affinity of at least about 10^8 M^{-1} . The antibody may be
30 monoclonal or may be a polyclonal composition, such as a polyclonal antisera. In a related aspect, the invention provides a cell capable of secreting the antibody, such as a

hybridoma.

The invention also provides a method for determining whether a compound or treatment is a modulator of a telomerase reverse transcriptase activity or hTERT expression. The method involves detecting or monitoring a change in activity or expression in a cell, animal or composition comprising an hTERT protein or polynucleotide following administration of the compound or treatment. In one embodiment, the method includes the steps of: providing a TRT composition, contacting the TRT with the test compound and measuring the activity of the TRT, where a change in TRT activity in the presence of the test compound is an indicator that the test compound modulates TRT activity. In certain embodiments, the composition is a cell, an organism, a transgenic organism or an *in vitro* system, such as an expression system, which contains a recombinant polynucleotide encoding an hTERT polypeptide. Thus, the hTERT of the method may be a product of *in vitro* expression. In various embodiments the detection of telomerase activity or expression may be by detecting a change in abundance of an hTERT gene product, monitoring incorporation of a nucleotide label into a substrate for telomerase, monitoring hybridization of a probe to an extended telomerase substrate, monitoring amplification of an extended telomerase substrate, monitoring telomere length of a cell exposed to the test compound, monitoring the loss of the ability of the telomerase to bind to a chromosome, or measuring the accumulation or loss of telomere structure.

In one aspect, the invention provides a method of detecting an hTERT gene product in a biological sample by contacting the biological sample with a probe that specifically binds the gene product, wherein the probe and the gene product form a complex, and detecting the complex, where the presence of the complex is correlated with the presence of the hTERT gene product in the biological sample. The gene product may be RNA, DNA or a polypeptide. Examples of probes that may be used for detection include, but are not limited to, nucleic acids and antibodies.

In one embodiment, the gene product is a nucleic acid which is detected by amplifying the gene and detecting the amplification product, where the presence of the complex or amplification product is correlated with the presence of the hTERT gene product in the biological sample.

In one embodiment, the biological sample is from a patient, such as a human patient. In another embodiment the biological sample includes at least one cell from an *in vitro* cell culture, such as a human cell culture.

5 The invention further provides a method of detecting the presence of at least one immortal or telomerase positive human cell in a biological sample comprising human cells by obtaining the biological sample comprising human cells; and detecting the presence in the sample of a cell having a high level of an hTERT gene product, where the presence of a cell having a high level of the hTERT gene product is correlated with the presence of immortal or telomerase positive cells in the biological sample.

10 The invention also provides a method for diagnosing a telomerase-related condition in a patient by obtaining a cell or tissue sample from the patient, determining the amount of an hTERT gene product in the cell or tissue; and comparing the amount of hTERT gene product in the cell or tissue with the amount in a healthy cell or tissue of the same type, where a different amount of hTERT gene product in the sample from the patient and the healthy cell or tissue is diagnostic of a telomerase-related condition. In one embodiment the telomerase-related condition is cancer and a greater amount of hTERT gene product is detected in the sample.

The invention further provides a method of diagnosing cancer in a patient by obtaining a biological sample from the patient, and detecting a hTERT gene product in the patient sample, where the detection of the hTERT gene product in the sample is correlated with a diagnosis of cancer.

20 The invention further provides a method of diagnosing cancer in a patient by obtaining a patient sample, determining the amount of hTERT gene product in the patient sample; and comparing the amount of hTERT gene product with a normal or control value, where an amount of the hTERT gene product in the patient that is greater than the normal or control value is diagnostic of cancer.

25 The invention also provides a method of diagnosing cancer in a patient by obtaining a patient sample containing at least one cell; determining the amount of an hTERT gene product in a cell in the sample; and comparing the amount of hTERT gene product in the cell with a normal value for the cell, wherein an amount of the hTERT gene product greater than the normal value is diagnostic of cancer. In one embodiment,

the sample is believed to contain at least one malignant cell.

The invention also provides a method for a prognosing a cancer patient by determining the amount of hTERT gene product in a cancer cell obtained from the patient; and comparing the amount of hTERT in the cancer cell with a prognostic value of hTERT consistent with a prognosis for the cancer; where an amount of hTERT in the sample that is at the prognostic value provides the particular prognosis.

The invention also provides a method for monitoring the ability of an anticancer treatment to reduce the proliferative capacity of cancer cells in a patient, by making a first measurement of the amount of an hTERT gene product in at least one cancer cell from the patient; making a second measurement of the level of the hTERT gene product in at least one cancer cell from the patient, wherein the anticancer treatment is administered to the patient before the second measurement; and comparing the first and second measurements, where a lower level of the hTERT gene product in the second measurement is correlated with the ability of an anticancer treatment to reduce the proliferative capacity of cancer cells in the patient.

The invention also provides kits for the detection of an hTERT gene or gene product. In one embodiment, the kit includes a container including a molecule selected from an hTERT nucleic acid or subsequence thereof, an hTERT polypeptide or subsequence thereof, and an anti-hTERT antibody.

The invention also provides methods of treating human diseases. In one embodiment, the invention provides a method for increasing the proliferative capacity of a vertebrate cell, such as a mammalian cell, by introducing a recombinant polynucleotide into the cell, wherein said polynucleotide comprises a sequence encoding an hTERT polypeptide. In one embodiment, the hTERT polypeptide has a sequence as shown in Figure 17. In one embodiment, the sequence is operably linked to a promoter. In one embodiment, the hTERT has telomerase catalytic activity. In one embodiment, the cell is human, such as a cell in a human patient. In an alternative embodiment, the cell is cultured *in vitro*. In a related embodiment, the cell is introduced into a human patient.

The invention further provides a method for treating a human disease by introducing recombinant hTERT polynucleotide into at least one cell in a patient. In one

embodiment, a gene therapy vector is used. In a related embodiment, the method further consists of introducing into the cell a polynucleotide comprising a sequence encoding hTR, for example, an hTR polynucleotide operably linked to a promoter.

The invention also provides a method for increasing the proliferative capacity of a vertebrate cell, said method comprising introducing into the cell an effective amount of hTERT polypeptide. In one embodiment the hTERT polypeptide has telomerase catalytic activity. The invention further provides cells and cell progeny with increased proliferative capacity.

The invention also provides a method for treating a condition associated with an elevated level of telomerase activity within a cell, comprising introducing into said cell a therapeutically effective amount of an inhibitor of said telomerase activity, wherein said inhibitor is an hTERT polypeptide or an hTERT polynucleotide. In one embodiment, the inhibitor is a polypeptide or polynucleotide comprising, e.g., at least a subsequence of a sequence shown in Figures 16, 17, or 20. In additional embodiments, the polypeptide or polynucleotide inhibits a TRT activity, such as binding of endogenous TRT to telomerase RNA.

The invention also provides a vaccine comprising an hTERT polypeptide and an adjuvant. The invention also provides pharmacological compositions containing a pharmaceutically acceptable carrier and a molecule selected from: an hTERT polypeptide, a polynucleotide encoding an hTERT polypeptide, and an hTERT nucleic acid or subsequence thereof.

DESCRIPTION OF THE FIGURES

Figure 1 shows highly conserved residues in TRT motifs from human, *S. pombe* (tez1), *S. cerevisiae* (EST2) and *Euplotes aediculatus* (p123). Identical amino acids are indicated with an asterisk (*) [raised slightly], while the similar amino acid residues are indicated by a dot (•). Motif "0" in the figure is also called Motif T; Motif "3" is also called Motif A.

Figure 2 shows the location of telomerase-specific and RT-specific sequence motifs of telomerase proteins and other reverse transcriptases. Locations of telomerase-specific motif T and conserved RT motifs 1, 2 and A-E are indicated by

boxes. The open rectangle labeled HIV-1 RT delineates the portion of this protein shown in Figure 3.

Figure 3 shows the crystal structure of the p66 subunit of HIV-1 reverse transcriptase (Brookhaven code 1HNV). The view is from the back of the right hand to enable all motifs to be shown.

Figure 4 shows multiple sequence alignment of telomerase RTs (Sp_Trt1p, *S. pombe* TRT [also referred to herein as "tez1p"]; hTRT, human TRT; Ea_p123, *Euplotes* p123; Sc_Est2p, *S. cerevisiae* Est2p) and members of other RT families (Sc_al, cytochrome oxidase group II intron 1-encoded protein from *S. cerevisiae* mitochondria, Dm_TART, reverse transcriptase from *Drosophila melanogaster* TART non-LTR retrotransposable element); HIV-1, human immunodeficiency virus reverse transcriptase). TRT con and RT con represent consensus sequences for telomerase RTs and non-telomerase RTs. Amino acids are designated with an h, hydrophobic; p, polar; c, charged. Triangles show residues that are conserved among telomerase proteins but different in other RTs. The solid line below motif E highlights the primer grip region.

Figure 5 shows expression of hTRT RNA in telomerase-negative mortal cell strains and telomerase-positive immortal cell lines as described in Example 2.

Figure 6 shows a possible phylogenetic tree of telomerases and retroelements rooted with RNA-dependent RNA polymerases.

Figure 7 shows a restriction map of lambda clone Gφ5.

Figure 8 shows a map of chromosome 5p with the location of the STS marker D5S678 (located near the hTRT gene) indicated.

Figure 9 shows the construction of a hTRT promoter-reporter plasmid.

Figure 10, in two pages, shows coexpression *in vitro* of hTRT and hTR to produce catalytically active human telomerase.

Figure 11, in two pages, shows an alignment of sequences from four TRT protein and identifies motifs of interest. TRT con shows a TRT consensus sequence. RT con shows consensus residues for other reverse transcriptases. Consensus residues in upper case indicate absolute conservation in TRT proteins.

Figure 12 shows a Topoisomerase II cleavage site and NFkB binding site motifs in an hTRT intron, with the sequence shown corresponding to SEQUENCE ID NO:7.

Figure 13, in two pages, shows the sequence of the DNA encoding the
5 *Euplotes* 123 kDa telomerase protein subunit (*Euplotes* TRT).

Figure 14 shows the amino acid sequence of the *Euplotes* 123 kDa telomerase protein subunit (*Euplotes* TRT protein).

Figure 15, in five pages, shows the DNA and amino acid sequences of the *S. pombe* telomerase catalytic subunit (*S. pombe* TRT).

10 Figure 16, in two pages, shows the hTRT cDNA sequence, with the sequence shown corresponding to SEQUENCE ID NO: 1.

Figure 17 shows the hTRT protein encoded by the cDNA of Figure 16. The protein sequence shown corresponds to SEQUENCE ID NO: 2.

15 Figure 18 shows the sequence of clone 712562, with the sequence shown corresponding to SEQUENCE ID NO: 3.

Figure 19 shows a 259 residue protein encoded by clone 712562, with the sequence shown corresponding to SEQUENCE ID NO 10.

20 Figure 20 shows, in seven pages, the sequence of a nucleic acid with an open reading frame encoding a Δ 182 variant polypeptide, with the sequence shown corresponding to SEQUENCE ID NO: 4. This Figure also shows the amino acid sequence of this Δ 182 variant polypeptide, with the amino acid sequence shown corresponding to SEQUENCE ID NO: 5.

25 Figure 21 shows, in six pages, sequence from an hTRT genomic clone, with the sequence shown corresponding to SEQUENCE ID NO: 6. Consensus motifs and elements are indicated, including sequences characteristic of a topoisomerase II cleavage site, NFkB binding sites, an Alu sequence and other sequence elements.

Figure 22 shows the effect of mutation of the TRT gene in yeast, as described in Example 1.

30 Figure 23 shows the sequence of EST AA281296, corresponding to SEQUENCE ID NO: 8.

Figure 24 shows the sequence of the 182 basepairs deleted in clone 712562, with the sequence shown corresponding to SEQUENCE ID NO: 9.

Figure 25 shows the results of an assay for telomerase activity from BJ cells transfected with an expression vector encoding an hTERT protein (pGRN133) or a control plasmid (pBBS212) as described in Example 13.

Figure 26 is a schematic diagram of the affinity purification of telomerase showing the binding and displacement elution steps.

Figure 27 is a photograph of a Northern blot of telomerase preparations obtained during a purification protocol, as described in Example 1. Lane 1 contained 1.5 fmol telomerase RNA, lane 2 contained 4.6 fmol telomerase RNA, lane 3 contained 14 fmol telomerase RNA, lane 4 contained 41 fmol telomerase RNA, lane 5 contained nuclear extract (42 fmol telomerase), lane 6 contained Affi-Gel-heparin-purified telomerase (47 fmol telomerase), lane 7 contained affinity-purified telomerase (68 fmol), and lane 8 contained glycerol gradient-purified telomerase (35 fmol).

Figure 28 shows telomerase activity through a purification protocol.

Figure 29 is a photograph of a SDS-PAGE gel, showing the presence of an approximately 123 kDa polypeptide and an approximately 43 kDa doublet from *Euplotes aediculatus*.

Figure 30 is a graph showing the sedimentation coefficient of *Euplotes aediculatus* telomerase.

Figure 31 is a photograph of a polyacrylamide/urea gel with 36% formamide showing the substrate utilization of *Euplotes* telomerase.

Figure 32 shows the putative alignments of telomerase RNA template and hairpin primers with telomerase RNA.

Figure 33 is a photograph of lanes 25-30 of the gel shown in Figure 31, shown at a lighter exposure level.

Figure 34 shows the DNA sequence of the gene encoding the 43 kDa telomerase protein subunit from *Euplotes*.

Figure 35 shows, in four pages, the DNA sequence, as well as the amino acid sequences of all three open reading frames of the 43 kDa telomerase protein subunit from *Euplotes*.

Figure 36 shows a sequence comparison between the 123 kDa telomerase protein subunit of *Euplotes* (upper sequence) and the 80 kDa polypeptide subunit of *T. thermophila* (lower sequence).

5 Figure 37 shows a sequence comparison between the 123 kDa telomerase protein subunit of *E. aediculatus* (upper sequence) and the 95 kDa telomerase polypeptide of *T. thermophila* (lower sequence).

Figure 38 shows the best-fit alignment between a portion of the "La-domain" of the 43 kDa telomerase protein subunit of *E. aediculatus* (upper sequence) and a portion of the 95 kDa polypeptide subunit of *T. thermophila* (lower sequence).

10 Figure 39 shows the best-fit alignment between a portion of the "La-domain" of the 43 kDa telomerase protein subunit of *E. aediculatus* (upper sequence) and a portion of the 80 kDa polypeptide subunit of *T. thermophila* (lower sequence).

Figure 40 shows the alignment and motifs of the polymerase domain of the 123 kDa telomerase protein subunit of *E. aediculatus* and the polymerase domains of various reverse transcriptases including a cytochrome oxidase group II intron 1-encoded protein from *S. cerevisiae* mitochondria (a1 S.c. (group II)), Dong (LINE), and yeast ESTp (L8543.12).

Figure 41 shows the alignment of a domain of the 43 kDa telomerase protein subunit with various La proteins.

20 Figure 42 shows the nucleotide sequence encoding the *T. thermophila* 80 kDa protein subunit.

Figure 43 shows the amino acid sequence of the *T. thermophila* 80 kDa protein subunit.

25 Figure 44 shows the nucleotide sequence encoding the *T. thermophila* 95 kDa protein subunit.

Figure 45 shows the amino acid sequence of the *T. thermophila* 95 kDa protein subunit.

Figure 46 shows the amino acid sequence of L8543.12 ("Est2p").

30 Figure 47 shows the alignment of the amino acid sequence encoded by the *Oxytricha* PCR product with the *Euplotes* p123 sequence.

Figure 48 shows the DNA sequence of Est2.

Figure 49 shows partial amino acid sequence from a cDNA clone encoding human telomerase peptide motifs.

Figure 50 shows partial DNA sequence of a cDNA clone encoding human telomerase peptide motifs.

5 Figure 51 shows the amino acid sequence of *tez1*, also called *S. pombe trt*.

Figure 52 shows, in two pages, the DNA sequence of *tez1*. Intronic and other non-coding regions are shown in lower case and exons (*i.e.*, coding regions) are shown in upper case.

10 Figure 53 shows the alignment of EST2p, *Euplotes*, and *Tetrahymena* sequences, as well as consensus sequence.

Figure 54 shows the sequences of peptides useful for production of anti-hTRT antibodies.

Figure 55 is a schematic summary of the *tez1* sequencing experiments.

15 Figure 56 shows two degenerate primers used in PCR to identify the *S. pombe* homolog of the *E. aedicularius* p123 sequences.

Figure 57 shows the four major bands produced in PCR using degenerate primers to identify the *S. pombe* homolog of the *E. aedicularius* p123 sequences.

20 Figure 58 shows the alignment of the M2 PCR product with *E. aedicularius* p123, *S. cerevisiae*, and *Oxytricha* telomerase protein sequences.

Figure 59 is a schematic showing the 3' RT PCR strategy for identifying the *S. pombe* homolog of the *E. aedicularius* p123.

25 Figure 60 shows characteristics of the libraries used to screen for *S. pombe* telomerase protein sequences and shows the results of screening the libraries for *S. pombe* telomerase protein sequences.

Figure 61 shows the positive results obtained with the *HindIII*-digested positive genomic clones containing *S. pombe* telomerase sequence.

30 Figure 62 is a schematic showing the 5' RT PCR strategy used to obtain a full length *S. pombe* TRT clone.

Figure 63 shows the alignment of RT domains from telomerase catalytic

subunits for *S. pombe* (S.p.), *S. cerevisiae* (S.c.) and *E. aediculatus* (E.a.).

Figure 64 shows the alignment of the sequences from *Euplotes* ("Ea_p123"), *S. cerevisiae* ("Sc_Est2p"), and *S. pombe* ("Sp_Tez1p"). In Panel A, the shaded areas indicate residues shared between two sequences. In Panel B, the shaded areas indicate residues shared between all three sequences.

Figure 65 shows the disruption strategy used with the telomerase genes in *S. pombe*.

Figure 66 shows the experimental results confirming disruption of *tez1*.

Figure 67 shows the progressive shortening of telomeres in *S. pombe* due to *tez1* disruption.

Figure 68 shows, in four pages, the DNA and amino acid of the ORF encoding an approximately 63 kDa telomerase protein or fragment thereof encoded the EcoRI-NotI insert of clone 712562.

Figure 69 shows an alignment of reverse transcriptase motifs from various sources.

Figure 70 provides a restriction and function map of plasmid pGRN121.

Figure 71 shows, in two pages, the results of preliminary nucleic acid sequencing analysis of a hTRT cDNA sequence.

Figure 72 shows, in ten pages, the preliminary nucleic acid sequence of hTRT and deduced ORF sequences in three reading frames.

Figure 73 provides a restriction and function map of plasmid pGRN121.

Figure 74 shows, in eight pages, refined nucleic acid sequence and deduced ORF sequences of hTRT

Figure 75 shows a restriction map of lambda clone 25-1.1.

DETAILED DESCRIPTION OF THE INVENTION

I. INTRODUCTION

Telomerase is a ribonucleoprotein complex (RNP) comprising an RNA component and a catalytic protein component. The present invention relates to the cloning and characterization of the catalytic protein component of telomerase, hereinafter referred to as "TRT" (telomerase reverse transcriptase). TRT is so named because this protein acts as an RNA-dependent DNA polymerase (reverse transcriptase), using the telomerase RNA component (hereinafter, "TR") to direct synthesis of telomere DNA repeat sequences. Moreover, TRT is evolutionarily related to other reverse transcriptases (see Example 12).

In one aspect, the present invention relates to the cloning and characterization of the catalytic protein component of human telomerase, hereinafter referred to as "hTRT." Human TRT is of extraordinary interest and value because, as noted *supra*, telomerase activity in human (and other mammalian cells) correlates with cell proliferative capacity, cell immortality, and the development of a neoplastic phenotype. For example, telomerase activity, and, as demonstrated in Example 2, *infra*, levels of human TRT gene products are elevated in immortal human cells (such as malignant tumor cells and immortal cell lines) relative to mortal cells (such as most human somatic cells).

The present invention further provides methods and compositions valuable for diagnosis, prognosis, and treatment of human diseases and disease conditions, as described in some detail *infra*. Also provided are methods and reagents useful for immortalizing cells (*in vivo* and *ex vivo*), producing transgenic animals with desirable characteristics, and numerous other uses, many of which are described *infra*. The invention also provides methods and reagents useful for preparing, cloning, or re-cloning TRT genes and proteins from ciliates, fungi, vertebrates, such as mammals, and other organisms.

As described in detail *infra*, TRT was initially characterized following purification of telomerase from the ciliate *Euplotes aediculatus*. Extensive purification of *E. aediculatus* telomerase, using RNA-affinity chromatography and other methods, yielded the protein "p123". Surprisingly, p123 is unrelated to

proteins previously believed to constitute the protein subunits of the telomerase holoenzyme (i.e., the p80 and p95 proteins of *Tetrahymena thermophila*). Analysis of the p123 DNA and protein sequences (Genbank Accession No. U95964; Figures 13 and 14) revealed reverse transcriptase (RT) motifs consistent with the role of p123 as the catalytic subunit of telomerase (see, e.g., Figures 1, 4 and 11). Moreover, p123 is related to a *S. cerevisiae* (yeast) protein, Est2p, which was known to play a role in maintenance of telomeres in *S. cerevisiae* (Genbank Accession No. S5396), but prior to the present invention was not recognized as encoding a telomerase catalytic subunit protein (see, e.g., Lendvay et al., 1996, *Genetics*, 144:1399).

In one aspect, the present invention provides reagents and methods for identifying and cloning novel TRTs using: nucleic acid probes and primers generated or derived from the TRT polynucleotides disclosed (e.g., for cloning TRT genes and cDNAs); antibodies that specifically recognize the motifs or motif sequences or other TRT epitopes (e.g., for expression cloning TRT genes or purification of TRT proteins); by screening computer databases; or other means. For example, as described in Example 1, PCR (polymerase chain reaction) amplification of *S. pombe* DNA was carried out with degenerate-sequence primers designed from the *Euplotes* p123 RT motifs B' and C. Of four prominent products generated, one encoded a peptide sequence homologous to *Euplotes* p123 and *S. cerevisiae* Est2p. Using this PCR product as a probe, the complete sequence of the *S. pombe* TRT homologue was obtained by screening of *S. pombe* cDNA and genomic libraries and amplifying *S. pombe* RNA by reverse transcription and PCR (RT-PCR). The complete sequence of the *S. pombe* gene ("trt1"; GenBank Accession No. AF015783; Figure 15) revealed that homology with p123 and Est2p was especially high in the reverse transcriptase motifs. *S. pombe* trt1 is also referred to as tez1.

Amplification using degenerate primers derived from the telomerase RT motifs was also used to obtain TRT gene sequences in *Oxytricha trifallax* and *Tetrahymena thermophila*, as described in Example 1.

The *Euplotes* p123, *S. pombe* trt1, and *S. cerevisiae* Est2p nucleic acid sequences of the invention were used in a search of a computerized database of human expressed sequence tags (ESTs) using the program BLAST (Altschul et al,

1990, *J. Mol. Biol.* 215:403). Searching this database with the Est2p sequence did not indicate a match, but searching with p123 and trt1 sequences identified a human EST (Genbank accession no. AA281296; see SEQUENCE ID NO: 8), as described in Example 1, putatively encoding a homologous protein. Complete sequencing of the cDNA clone containing the EST (hereinafter, "clone 712562"; see SEQUENCE ID NO: 3) showed that seven RT motifs were present. However, this clone did not encode a contiguous human TRT with all seven motifs, because motifs B', C, D, and E were contained in a different open reading frame (ORF) than the more NH₂-terminal motifs. In addition, the distance between motifs A and B' was substantially shorter than that of the three previously characterized TRTs. Clone 712562 was obtained from the I.M.A.G.E. Consortium; Lennon et al., 1996, *Genomics* 33:151.

A cDNA clone, pGRN121, encoding a functional hTRT (see Figure 16, SEQUENCE ID NO: 1) was isolated from a cDNA library derived from the human 293 cell line as described in Example 1. Comparing clone 712562 with pGRN121 showed that clone 712562 has a 182 base pair (see Figure 24, SEQUENCE ID NO: 9) deletion between motifs A and B'. The additional 182 base pairs present in pGRN121 place all of the TRT motifs in a single open reading frame, and increase the spacing between the motif A and motif B' regions to a distance consistent with the other known TRTs. As is described *infra* in the Examples (e.g., Example 7), SEQUENCE ID NO: 1 encodes a catalytically active telomerase protein having the sequence of SEQUENCE ID NO: 2. The polypeptide of SEQUENCE ID NO: 2 has 1132 residues and a calculated molecular weight of about 127 kilodaltons (kD).

As is discussed *infra*, and described in Example 9, *infra*, TRT cDNAs possessing the 182 basepair deletion characteristic of the clone 712562 are detected following reverse transcription of RNA from telomerase-positive cells (e.g., testis and 293 cells). hTRT RNAs lacking this 182 base pair sequence are referred to generally as "Δ182 variants" and may represent one, two, or several species. Although the hTRT variants lacking the 182 basepair sequence found in the pGRN121 cDNA are unlikely to encode a fully active telomerase catalytic enzyme, they may play a role in telomerase regulation, as discussed *infra*, and/or have partial telomerase activity, such as telomere binding or hTR binding activity, as discussed *infra*.

Thus, in one aspect, the present invention provides an isolated polynucleotide with a sequence of a naturally occurring human TRT gene or mRNA including, but not limited to, a polynucleotide having the sequence as set forth in Figure 16 (SEQUENCE ID NO: 1). In a related aspect, the invention provides a
5 polynucleotide encoding an hTRT protein, fragment, variant or derivative. In another related aspect, the invention provides sense and antisense nucleic acids that bind to an hTRT gene or mRNA. The invention further provides hTRT proteins, whether synthesized or purified from natural sources, as well as antibodies and other agents that specifically bind an hTRT protein or a fragment thereof. The present invention also
10 provides many novel methods, including methods that employ the aforementioned compositions, for example, by providing diagnostic and prognostic assays for human diseases, methods for developing therapeutics and methods of therapy, identification of telomerase-associated proteins, and methods for screening for agents capable of activating or inhibiting telomerase activity. Numerous other aspects and embodiments
15 of the invention are provided *infra*.

One aspect of the invention is the use of a polynucleotide that is at least ten nucleotides to about 10 kb or more in length and comprises a contiguous sequence of at least ten nucleotides that is identical or exactly complementary to a contiguous sequence in a naturally occurring hTRT gene or hTRT mRNA in assaying or screening for an hTRT gene sequence or
20 hTRT mRNA, or in preparing a recombinant host cell.

A further aspect of the invention is the use of an agent increasing expression of hTRT in the manufacture of a medicament for the treatment of a condition addressed by increasing proliferative capacity of a vertebrate cell, optionally the medicament being for inhibiting the effects of ageing.

25 Yet a further aspect of the invention is the use of an inhibitor of telomerase activity in the manufacture of a medicament for the treatment of a condition associated with an elevated level of telomerase activity within a human cell.

The proteins, variants and fragments of the invention, and the encoding polynucleotides or fragments, are also each provided in a further aspect of this invention for use
30 as a pharmaceutical.

The invention further includes the use of a protein, variant or fragment, or of a polynucleotide or fragment, in each case as defined herein, in the manufacture of a medicament, for example in the manufacture of a medicament for inhibiting an effect of aging or cancer.

In certain embodiments of the present invention, the hTRT polynucleotides are other than the 389 nucleotide polynucleotide of SEQUENCE ID NO:8 and/or other than clone 712562, the plasmid containing an insert, the sequence of which insert is shown in Figure 18 (SEQUENCE ID NO:3).

Another aspect of the present invention is a polynucleotide selected from:

(a) the DNA having a sequence as set forth in Figure 16;

(b) a polynucleotide of at least 10 nucleotides which hybridizes to the foregoing DNA and which codes for an hTRT protein or variant;

(c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b) and which code for an hTRT polypeptide or variant.

The description below is organized by topic. Part II further describes amino acid motifs characteristic of TRT proteins, as well as TRT genes encoding proteins having such motifs. Parts III-VI describe, *inter alia*, nucleic acids, proteins, antibodies and purified compositions of the invention with particular focus on human TRT related compositions. Part VII describes, *inter alia*, methods and compositions of the invention useful for treatment of human disease. Part VIII describes production and identification of immortalized human cell lines. Part IX describes, *inter alia*, uses of the nucleic acids, polynucleotides, and other compositions of the invention for diagnosis of human diseases. Part X describes, *inter alia*, methods and compositions of the invention useful for screening and identifying agents and treatments that modulate (e.g., inhibit or promote) telomerase activity or expression. Part XI describes, *inter alia*, transgenic animals (e.g., telomerase knockout animals and cells). Part XII is a glossary of terms used in Parts I-XI. Part XIII describes examples relating to specific embodiments of the invention. The organization of the description of the invention by topic and subtopic is to provide clarity, and not to be limiting in any way.

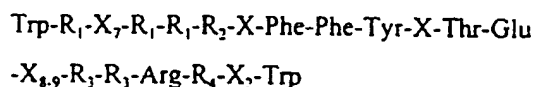
II. TRT GENES AND PROTEINS

The present invention provides isolated and/or recombinant genes and proteins having a sequence of a telomerase catalytic subunit protein (i.e., telomerase reverse transcriptase), including, but not limited to, the naturally occurring forms of such genes and proteins in isolated or recombinant form. Typically, TRTs are large, basic, proteins having *reverse transcriptase* (RT) and *telomerase-specific* (T) amino acid motifs, as disclosed herein. Because these motifs are conserved across diverse organisms, TRT genes of numerous organisms may be obtained using the methods of the invention or identified using primers, nucleic acid probes, and antibodies of the invention, such as those specific for one or more of the motif sequences.

The seven RT motifs found in TRTs, while similar to those found in other reverse transcriptases, have particular hallmarks. For example, as shown in Figure 4, within the TRT RT motifs there are a number of amino acid substitutions (marked with arrows) in residues highly conserved among the other RTs. For example, in motif C the two aspartic acid residues (DD) that coordinate active site metal ions (see, Kohlstaedt et al., 1992, *Science* 256:1783; Jacobo-Molina et al., 1993, *Proc Natl Acad Sci. U.S.A.* 90:6320; Patel et al., 1995, *Biochemistry* 34:5351) occur in the context hxDD(F/Y) in the telomerase RTs compared to (F/Y)xDDh in the other RTs (where "h" is a hydrophobic amino acid, and "x" is any amino acid; see Xiong et al., 1990, *EMBO J.* 9:3353; Eickbush, in *The Evolutionary Biology of Viruses*, (S. Morse, Ed., Raven Press, NY, p. 121, 1994)). Another systematic change characteristic of the telomerase subgroup occurs in motif E, where WxGxSx is a consensus sequence or is conserved among the telomerase proteins, whereas hLGxxh is characteristic of other RTs (Xiong et al., *supra*; Eickbush *supra*). This motif E is called the "primer grip", and mutations in this region have been reported to affect RNA priming but not DNA priming (Powell et al., 1997, *J. Biol. Chem.* 272:13262). Because telomerase requires a DNA primer (e.g., the chromosome 3' end), it is not unexpected that telomerase should differ from other RTs in the primer grip region. In addition, the distance between motifs A and B' is longer in the TRTs than is typical for other RTs, which may represent an insertion within the "fingers" region of the structure which resembles a right hand (Figure 3; see Kohlstaedt et al., *supra*; Jacobo-Molina et al., *supra*; and

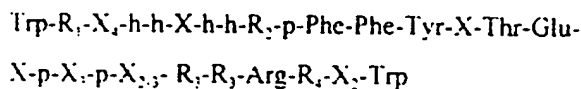
Patel et al., *supra*).

Moreover, as noted *supra*, Motif T is an additional hallmark of TRT proteins. This Motif T, as shown, for example in Figure 4 (W-L-X-Y-X-X-h-h-X-h-h-X-p-F-F-T-E-X-p-X-X-X-p-X-X-X-Y-X-R-K-X-X-W [X is any amino acid, h is hydrophobic, p is polar]), comprises a sequence that can be described using the formula:



where X is any amino acid and the subscript refers to the number of consecutive residues, R₁ is leucine or isoleucine, R₂ is glutamine or arginine, R₃ is phenylalanine or tyrosine, and R₄ is lysine or histidine.

The T motif can also be described using the formula:



where X is any amino acid and a subscript refers to the number of consecutive residues. R₁ is leucine or isoleucine, R₂ is glutamine or arginine, R₃ is phenylalanine or tyrosine, R₄ is lysine or histidine, h is a hydrophobic amino acid selected from Ala, Leu, Ile, Val, Pro, Phe, Trp, and Met, and p is a polar amino acid selected from Gly, Ser, Thr, Tyr, Cys, Asn and Gln.

In one embodiment, the present invention provides isolated naturally occurring and recombinant TRT proteins comprising one or more of the motifs illustrated in Figure 11, e.g.,

| | |
|----------|---|
| Motif T | W-X ₁₂ -FFY-X-TE-X ₁₀₋₁₁ -R-X ₃ -W-X ₇ -I |
| Motif T' | E-X ₂ -V-X |
| Motif 1 | X ₃ -R-X ₂ -PK-X ₃ |
| Motif 2 | X-R-X-I-X |
| Motif A | X ₄ -F-X ₃ -D-X ₄ -YD-X ₂ |
| Motif B' | Y-X ₄ -G-X ₂ -QG-X ₃ -S-X ₃ |
| Motif C | X ₆ -DD-X-L-X ₃ |

When the TRT protein shown contains more than one TRT motif, the order (NH₂ - >COOH) is as shown in Figure 11.

In one embodiment, the present invention provides isolated naturally occurring TRT proteins comprising the following supermotif:

(NH₂)- X₃₀₀₋₆₀₀-W-X₁₂-FFY-X-TE-X₁₀₋₁₁-R-X₃-W-X₇-I-X₅₋₂₀-E-X₂-V-X-X₅₋₂₀-X₃-R-X₂-
PK-X₄₋₁₀-R-X-I-X-X₆₀₋₈₀-X₄-F-X₃-D-X₄-YD-X₂-X₈₀₋₁₃₀-Y-X₄-G-X₂-QG-X₃-S-X₆-X₅₋₃₅-
X₅-DD-X-L-X₃-X₁₀₋₂₀-X₁₂-K

It will be apparent to one of skill that, provided with the reagents, including the TRT sequences disclosed herein for those reagents and the methods and guidance provided herein (including specific methodologies described *infra*), TRT genes and proteins can be obtained, isolated and produced in recombinant form by one of ordinary skill. For example, primers (e.g., degenerate amplification primers) are provided that hybridize to gene sequences encoding RT and T motifs characteristic of TRT. For example, one or more primers or degenerate primers that hybridize to sequences encoding the FFYXTE region of the T motif, other TRT motifs (as discussed *infra*), or combinations of motifs or consensus sequences, can be prepared based on the codon usage of the target organism, and used to amplify the TRT gene sequence from genomic DNA or cDNA prepared from the target organism. Use of degenerate primers is well known in the art and entails use of sets of primers that hybridize to the set of nucleic acid sequences that can potentially encode the amino acids of the target motif, taking into account codon preferences and usage of the target organism, and by using amplification (e.g., PCR) conditions appropriate for allowing base mismatches in the annealing steps of PCR. Typically two primer sets are used; however, single primer (or, in this case, a single degenerate primer set) amplification systems are well known and may be used to obtain TRT genes.

Table 1 provides illustrative primers of the invention that may be used to amplify novel TRT nucleic acids, particularly those from vertebrates (e.g., humans and other mammals). "N" is an equimolar mixture of all four nucleotides, and nucleotides within parentheses are equimolar mixtures of the specified nucleotides.

TABLE 1
ILLUSTRATIVE DEGENERATE PRIMERS FOR AMPLIFICATION
OF TRT NUCLEIC ACIDS

| | <u>motif</u> | <u>direction</u> | <u>5'- sequence -3'</u> |
|----|-----------------|------------------|-------------------------------------|
| 5 | | | |
| | a <u>FFYVIE</u> | Forward | TT(CT)TT(CT)TA(CT)GTNACNGA |
| 10 | b <u>FFYVIE</u> | Reverse | TCNGTNAC(GA)TA(GA)AA(GA)AA |
| | c <u>RFIPKP</u> | Forward | (CA)GN TT(CT)AT(ACT)CCNAA(AG)CC |
| | d <u>RFIPKP</u> | Reverse | GG(TC)TTNGG(TGA)AT(GA)AANC |
| 15 | e <u>AYDTI</u> | Forward | GCNTA(CT)GA(CT)ACNAT |
| | f <u>AYDTI</u> | Reverse | TANGT(GA)TC(GA)TANGC |
| | g <u>GIPOG</u> | Forward | GGNAT(ACT)CCNCA(AG)GG |
| | h <u>GIPOGS</u> | Reverse | (GC)ATNCC(TC)TGNGG(TGA)ATNCC |
| 20 | i <u>LVDDFL</u> | Forward | (CT)TNGTNGA(CT)GA(CT)TT(CT)(CT)T |
| | j <u>DDFLLV</u> | Reverse | GTNACNA(GA)NA(GA)GA(AA)GA(TC)GA(TC) |

Preferred primer combinations (y = yes, n = no)

| | | | | | | |
|----|----------------|----------------|----------|----------|----------|----------|
| 25 | | <u>Reverse</u> | | | | |
| | <u>Forward</u> | <u>b</u> | <u>d</u> | <u>f</u> | <u>h</u> | <u>j</u> |
| | a- | n | y | y | y | y |
| | c- | n | n | y | y | y |
| | e- | n | n | n | y | y |
| 30 | g- | n | n | n | n | y |
| | i- | n | n | n | n | n |

In one embodiment, an amplified TRT nucleic acid is used as a hybridization probe for colony hybridization to a library (e.g., cDNA library) made from the target organism, such that a nucleic acid having the entire TRT protein coding sequence, or a substantial portion thereof, is identified and isolated or cloned. Reagents and methods such as those just described were used in accordance with the methods described herein to obtain TRT gene sequences of *Oxytricha trifallax* and *Tetrahymena thermophila*, as described in detail *infra*. It will be recognized that following cloning of a previously uncharacterized TRT gene, the sequence can be determined by routine methods and the encoded polypeptide synthesized and assayed for a TRT activity, such as telomerase catalytic activity (as described herein and/or by telomerase assays known in the art).

It will also be apparent to those of skill that TRT genes may be cloned using any of a variety of cloning methods of the invention because the TRT motif sequences and the nucleic acids of the invention comprising such sequences can be used in a wide variety of such methods. For example, hybridization using a probe based on the sequence of a known TRT to DNA or other nucleic acid libraries from the target organism, as described in Example 1 can be used. It will be appreciated that degenerate PCR primers or their amplification products such as those described *supra*, may themselves be labeled and used as hybridization probes. In another embodiment, expression cloning methods are used. For example, one or more antibodies that specifically bind peptides that span a TRT motif or other TRT epitope, such as the FFYXTE motif can be employed to isolate a ribosomal complex comprising a TRT protein and the mRNA that encodes it. For generating such antibodies of the invention, the peptide immunogens are typically between 6 and 30 amino acids in length, more often about 10 to 20 amino acids in length. The antibodies may also be used to probe a cDNA expression library derived from the organism of interest to identify a clone encoding a TRT sequence. In another embodiment, computer searches of DNA databases for DNAs containing sequences conserved with known TRTs can also be used to identify a clone comprising TRT sequence.

In one aspect, the present invention provides compositions comprising an isolated or recombinant polypeptide having the amino acid sequence of a naturally

occurring TRT protein. Usually the naturally occurring TRT has a molecular weight of between about 80,000 daltons (D) and about 150,000 D, most often between about 95,000 D and about 130,000 D. Typically, the naturally occurring TRT has a net positive charge at pH 7 (calculated pI typically greater than 9). In one embodiment, the polypeptide exhibits a telomerase activity as defined herein. In a related embodiment, the polypeptide has a TRT-specific region (T motif) sequence and exhibits a telomerase activity. The invention further provides fragments of such polypeptides. The present invention also provides isolated or recombinant polynucleotide having the sequence of a naturally occurring gene encoding a TRT protein. The invention provides reagents useful for isolating sequence of a TRT from nonvertebrate (such as a yeast) and vertebrates, such as mammals (e.g., murine or human). The isolated polynucleotide may be associated with other naturally occurring or recombinant or synthetic vector nucleic acid sequences. Typically, the isolated nucleic acid is smaller than about 300 kb, often less than about 50 kb, more often less than about 20 kb, frequently less than about 10 kb and sometimes less than about 5 kb or 2 kb in length. In some embodiments the isolated TRT polynucleotide is even smaller, such as a gene fragment, primer, or probe of less than about 1 kb or less than 0.1 kb.

III. NUCLEIC ACIDS

A) GENERALLY

The present invention provides isolated and recombinant nucleic acids having a sequence of a polynucleotide encoding a telomerase catalytic subunit protein (TRT), such as a recombinant TRT gene from *Euplotes*, *Tetrahymena*, *S. pombe* or humans. Exemplary polynucleotides are provided in Figure 13 (*Euplotes*); Figure 15 (*S. pombe*) and Figure 16 (human, GenBank Accession No. AF015950). The present invention provides sense and anti-sense polynucleotides having a TRT gene sequence, including probes, primers, TRT-protein-encoding polynucleotides, and the like.

B) HUMAN TRT

The present invention provides nucleic acids having a sequence of a telomerase catalytic subunit from humans (i.e., hTRT).

In one aspect, the invention provides a polynucleotide having a sequence or subsequence of a human TRT gene or RNA. In one embodiment, the polynucleotide of the invention has a sequence of SEQUENCE ID NO: 1 shown in Figure 16 or a subsequence thereof. In another embodiment, the polynucleotide has a sequence of SEQUENCE ID NO: 3 (Figure 18), SEQUENCE ID NO: 4 (Figure 20), or subsequences thereof. The invention also provides polynucleotides with substantial sequence identity to the hTRT nucleic acid sequences disclosed herein, e.g., including but not limited to SEQUENCE ID NOS: 1 [Figure 16], 4 [Figure 20], 6 [Figure 21], and 7). Thus, the invention provides naturally occurring alleles of human TRT genes and variant polynucleotide sequences having one or more nucleotide deletions, insertions or substitutions relative to an hTRT nucleic acid sequence disclosed herein. As described *infra*, variant nucleic acids may be produced using the recombinant or synthetic methods described below or by other means.

The invention also provides isolated and recombinant polynucleotides having a sequence from a flanking region of a human TRT gene. Such polynucleotides include those derived from genomic sequences of untranslated regions of the hTRT mRNA. An exemplary genomic sequence is shown in Figure 21 (SEQUENCE ID NO: 6). As described in Example 4, SEQUENCE ID NO: 6 was obtained by sequencing a clone, λ G Φ 5 isolated from a human genomic library. Lambda G Φ 5 contains a 15 kilobasepair (kbp) insert including approximately 13,000 bases 5' to the hTRT coding sequences. This clone contains hTRT promoter sequences and other hTRT gene regulatory sequences (e.g., enhancers).

The invention also provides isolated and recombinant polynucleotides having a sequence from an intronic region of a human TRT gene. An exemplary intronic sequence is shown in Figure 21 (SEQUENCE ID NO: 7; see Example 3). In some embodiments, hTRT introns are included in "minigenes" for improved expression of hTRT proteins in eukaryotic cells.

In a related aspect, the present invention provides polynucleotides that encode hTRT proteins or protein fragments, including modified, altered and variant hTRT polypeptides. In one embodiment, the encoded hTRT protein or fragment has an amino acid sequence as set forth in Figure 17 (SEQUENCE ID NO: 2), or with

conservative substitutions of SEQUENCE ID NO: 2. In one embodiment, the encoded hTERT protein or fragment has substitutions that change an activity of the protein (e.g., telomerase catalytic activity).

It will be appreciated that, as a result of the degeneracy of the genetic code, the nucleic acid encoding the hTERT protein need not have the sequence of a naturally occurring hTERT gene, but that a multitude of polynucleotides can encode an hTERT polypeptide having an amino acid sequence of SEQUENCE ID NO: 2. The present invention provides each and every possible variation of nucleotide sequence that could be made by selecting combinations based on possible codon choices made in accordance with known triplet genetic codes, and all such variations are specifically disclosed hereby. Thus, although in some cases hTERT polypeptide-encoding nucleotide sequences that are capable of hybridizing to the nucleotide sequence of the naturally occurring sequence (under appropriately selected conditions of stringency) are preferred, it may be advantageous in other cases to produce nucleotide sequences encoding hTERT that employ a substantially different codon usage and so perhaps do not hybridize to nucleic acids with the naturally occurring sequence.

In particular embodiments, the invention provides hTERT oligo- and polynucleotides that comprise a subsequence of an hTERT nucleic acid disclosed herein (e.g., SEQUENCE ID NOS: 1 and 6). The nucleic acids of the invention typically comprise at least about 10, more often at least about 12 or about 15 consecutive bases of the exemplified hTERT polynucleotide. Often, the nucleic acid of the invention will comprise a longer sequence, such as at least about 25, about 50, about 100, about 200, or at least about 500 to 3000 bases in length, for example when expression of a polypeptide, or full length hTERT protein is intended.

In still other embodiments, the present invention provides "Δ182 hTERT" polynucleotides having a sequence identical or complementary to naturally occurring or non-naturally occurring hTERT polynucleotides such as SEQUENCE ID NO: 3 or SEQUENCE ID NO: 4, which do not contain the 182 nucleotide sequence (SEQUENCE ID NO: 9 [Figure 24]) found in pGRN121 (and also absent in clone 712562). These polynucleotides are of interest, in part, because they encode polypeptides that contain different combinations or arrangements of TRT motifs than

found in the "full-length" hTERT polypeptide (SEQUENCE ID NO: 2) such as is encoded by pGRN121. As discussed *infra*, it is contemplated that these polypeptides may play a biological role in nature (e.g., in regulation of telomerase expression in cells) and/or find use as therapeutics (e.g., as dominant-negative products that inhibit function of wild-type proteins), or have other roles and uses, e.g. as described herein.

For example, in contrast to the polypeptide encoded by pGRN121, clone 712562 encodes a 259 residue protein with a calculated molecular weight of approximately 30 kD (hereinafter, "712562 hTERT"). The 712562 hTERT polypeptide (SEQUENCE ID NO: 10 [Figure 19]) contains motifs T, 1, 2, and A, but not motifs B', C, D and E (See Figure 4). Similarly, a variant hTERT polypeptide with therapeutic and other activities may be expressed from a nucleic acid similar to the pGRN121 cDNA but lacking the 182 basepairs missing in clone 712562, e.g., having the sequence shown in Figure 20 (SEQUENCE ID NO: 4). This nucleic acid (hereinafter, "pro90 hTERT"), which may be synthesized using routine synthetic or recombinant methods as described herein, encodes a protein of 807 residues (calculated molecular weight of approximately 90 kD) that shares the same amino terminal sequence as the hTERT protein encoded by SEQUENCE ID NO: 1, but diverges at the carboxy-terminal region (the first 763 residues are common, the last 44 residues of pro90 hTERT are different than "full-length" hTERT). The pro90 hTERT polypeptide contains motifs T, 1, 2, and A, but not motifs B, C, D, E, and thus may have some, but not likely all telomerase activities.

C) PRODUCTION OF HUMAN TRT NUCLEIC ACIDS

The polynucleotides of the invention have numerous uses including, but not limited to, expression of polypeptides encoding hTERT or fragments thereof, use as sense or antisense probes or primers for hybridization and/or amplification of naturally occurring hTERT genes or RNAs (e.g. for diagnostic or prognostic applications), and as therapeutic agents (e.g., in antisense, triplex, or ribozyme compositions). As will be apparent upon review of the disclosure, these uses will have enormous impact on the diagnosis and treatment of human diseases relating to aging, cancer, and fertility as well as the growth, reproduction, and manufacture of cell-based products. As described in

the following sections, the hTERT nucleic acids of the invention may be made (e.g., cloned, synthesized, or amplified) using techniques well known in the art.

1) CLONING, AMPLIFICATION, AND RECOMBINANT

5 PRODUCTION

In one embodiment, hTERT genes or cDNAs are cloned using a nucleic acid probe that specifically hybridizes to an hTERT mRNA, cDNA, or genomic DNA. One suitable probe for this purpose is a polynucleotide having all or part of the sequence provided in Figure 16 (SEQUENCE ID NO: 1), such as a probe comprising a
10 subsequence thereof. Typically, the target hTERT genomic DNA or cDNA is ligated into a vector (e.g., a plasmid, phage, virus, yeast artificial chromosome, or the like) and may be isolated from a genomic or cDNA library (e.g., a human placental cDNA library). Once an hTERT nucleic acid is identified, it can be isolated according to standard methods known to those of skill in the art. An illustrative example of
15 screening a human cDNA library for the hTERT gene is provided in Example 1: similarly, an example of screening a human genomic library is found in Examples 3 and 4. Cloning methods are well known and are described, for example, in Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Vols 1-3, Cold Spring Harbor Laboratory (hereinafter, "Sambrook"); Berger and Kimmel (1987) Methods in
20 Enzymology, Vol. 152: Guide to Molecular Cloning Techniques, San Diego: Academic Press, Inc; Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York (1997); Cashion et al., US Patent No 5,017,478; and Carr, European Patent No 0 246 864.

The invention also provides hTERT genomic or cDNA nucleic acids
25 isolated by amplification methods such as the polymerase chain reaction (PCR). In one embodiment, hTERT protein coding sequence is amplified from an RNA or cDNA sample (e.g., double stranded placental cDNA (Clontech, Palo Alto CA)) using the primers 5'-GTGAAGGCACTGTTTCAGCG-3' ("TCP1.1") and
30 5'-CGCGTGGGTGAGGTGAGGTG-3' ("TCP 1.15"). In some embodiments a third primer or second pair of primers may be used, e.g., for "nested PCR", to increase

Specificity. One example of a second pair of primers is 5'-
CTGTGCTGGGCCTGGACGATA-3' ("billTCP6") and 5'-
AGCTTGTTCTCCATGTCGCCGTAG-3' ("TCP1.14"). It will be apparent to those
of skill that numerous other primers and primer combinations, useful for amplification
of hTERT nucleic acids are provided by the present invention.

5

10

15

20

25

30

Moreover, the invention provides primers that amplify any specific region (e.g., coding regions, promoter regions, and/or introns) or subsequence of hTRT genomic DNA, cDNA or RNA. For example, the hTRT intron at position 274/275 of SEQUENCE ID NO: 1 (see Example 3) may be amplified (e.g., for detection of genomic clones) using primers TCP1.57 and TCP1.52 (primer pair 1) or primers TCP1.49 and TCP1.50 (primer pair 2). (Primer names refer to primers listed in Table 2, *infra*.) The primer pairs can be used individually or in a nested PCR where primer set 1 is used first. Another illustrative example relates to primers that specifically amplify and so detect the 5' end of the hTRT mRNA or the exon encoding the 5' end of hTRT gene (e.g., to assess the size or completeness of a cDNA clone). The following primer pairs are useful for amplifying the 5' end of hTRT: primers K320 and K321 (primer pair 3); primers K320 and TCP1.61 (primer pair 4); primers K320 and K322 (primer pair 5). The primer sets can be used in a nested PCR in the order set 5, then set 4 or set 3, or set 4 or set 5, then set 3. Yet another illustrative example involves primers chosen to amplify or detect specifically the conserved hTRT TRT motif region comprising approximately the middle third of the mRNA (e.g., for use as a hybridization probe to identify TRT clones from, for example, nonhuman organisms). The following primer pairs are useful for amplifying the TRT motif region of hTRT nucleic acids: primers K304 and TCP1.8 (primer pair 6), or primers LT1 and TCP1.15 (primer pair 7). The primer sets can be used in a nested PCR experiment in the order set 6 then set 7.

Suitable PCR amplification conditions are known to those of skill and include (but are not limited to) 1 unit Taq polymerase (Perkin Elmer, Norwalk CT), 100 μ M each dNTP (dATP, dCTP, dGTP, dTTP), 1x PCR buffer (50 mM KCl, 10 mM Tris, pH 8.3 at room temperature, 1.5 mM MgCl₂, 0.01% gelatin) and 0.5 μ M primers, with the amplification run for about 30 cycles at 94° for 45 sec, 55° for 45 sec and 72° for 90 sec. It will be recognized by those of skill in the art that other thermostable DNA polymerases, reaction conditions, and cycling parameters will also provide suitable amplification. Other suitable *in vitro* amplification methods that can be used to obtain hTRT nucleic acids include, but are not limited to, those herein, *infra*. Once amplified, the hTRT nucleic acids can be cloned, if desired, into any of a variety of

vectors using routine molecular biological methods or detected or otherwise utilized in accordance with the methods of the invention.

One of skill will appreciate that the cloned or amplified hTERT nucleic acids obtained as described above can be prepared or propagated using other methods, such as chemical synthesis or replication by transformation into bacterial systems, such as *E. coli* (see, e.g., Ausubel et al., *supra*), or eukaryotic, such as mammalian, expression systems. Similarly, hTERT RNA can be expressed in accordance with the present *in vitro* methods, or in bacterial systems such as *E. coli* using, for example, commercially available vectors containing promoters recognized by an RNA polymerase such as T7, T3 or SP6, or transcription of DNA generated by PCR amplification using primers containing an RNA polymerase promoter.

The present invention further provides altered or modified hTERT nucleic acids. It will be recognized by one of skill that the cloned or amplified hTERT nucleic acids obtained can be modified (e.g., truncated, derivatized, altered) by methods well known in the art (e.g., site-directed mutagenesis, linker scanning mutagenesis) or simply synthesized *de novo* as described below. The altered or modified hTERT nucleic acids are useful for a variety of applications, including, but not limited to, facilitating cloning or manipulation of an hTERT gene or gene product, or expressing a variant hTERT gene product. For example, in one embodiment, the hTERT gene sequence is altered such that it encodes an hTERT polypeptide with altered properties or activities, as discussed in detail in *infra*, for example, by mutation in a conserved motif of hTERT. In another illustrative example, the mutations in the protein coding region of an hTERT nucleic acid may be introduced to alter glycosylation patterns, to change codon preference, to produce splice variants, remove protease-sensitive sites, create antigenic domains, modify specific activity, and the like. In other embodiments, the nucleotide sequence encoding hTERT and its derivatives is changed without altering the encoded amino acid sequences, for example, the production of RNA transcripts having more desirable properties, such as increased translation efficiency or a greater or a shorter half-life, compared to transcripts produced from the naturally occurring sequence. In yet another embodiment, altered codons are selected to increase the rate at which expression of the peptide occurs in a particular prokaryotic or eukaryotic expression

host in accordance with the frequency with which particular codons are utilized by the host. Useful *in vitro* and *in vivo* recombinant techniques that can be used to prepare variant hTRT polynucleotides of the invention are found in Sambrook et al. and Ausubel et al., both *supra*.

5 As noted *supra*, the present invention provides nucleic acids having flanking (5' or 3') and intronic sequences of the hTRT gene. The nucleic acids are of interest, *inter alia*, because they contain promoter and other regulatory elements involved in hTRT regulation and useful for expression of hTRT and other recombinant proteins or RNA gene products. It will be apparent that, in addition to the nucleic acid
10 sequences provided in SEQUENCE ID NOS: 6 and 7, additional hTRT intron and flanking sequences may be readily obtained using routine molecular biological techniques. For example, additional hTRT genomic sequence may be obtained from Lambda clone GΦ5 (ATCC Accession No. 209024), described *supra* and in Example 4. Still other hTRT genomic clones and sequences may be obtained by screening a human
15 genomic library using an hTRT nucleic acid probe having a sequence or subsequence from SEQUENCE ID NO: 1. Additional clones and sequences (e.g., still further upstream) may be obtained by using labeled sequences or subclones derived from λGΦ5 to probe appropriate libraries. Other useful methods for further characterization of hTRT flanking sequences include those general methods described by Gobinda et al.,
20 1993, *PCR Meth. Applic.* 2:318; Triglia et al., 1988, *Nucleic Acids Res.* 16:8186; Lagerstrom et al., 1991, *PCR Methods Applic.* 1:111; and Parker et al., 1991, *Nucleic Acids Res.* 19:3055.

Intronic sequences can be identified by routine means such as by comparing the hTRT genomic sequence with hTRT cDNA sequences (see, e.g.,
25 Example 3), by S1 analysis (see Ausubel et al., *supra*, at Chapter 4), or various other means known in the art. Intronic sequences can also be found in pre-mRNA (i.e., unspliced or incompletely spliced mRNA precursors), which may be amplified or cloned following reverse transcription of cellular RNA.

When desired, the sequence of the cloned, amplified, or otherwise
30 synthesized hTRT or other TRT nucleic acid can be determined or verified using DNA sequencing methods well known in the art (see, e.g., Ausubel et al., *supra*). Useful

methods of sequencing employ such enzymes as the Klenow fragment of DNA polymerase I, Sequenase (US Biochemical Corp, Cleveland OH), *Taq* DNA polymerase (Perkin Elmer, Norwalk CT), thermostable T7 polymerase (Amersham, Chicago IL), or combinations of recombinant polymerases and proofreading exonucleases such as the ELONGASE Amplification System marketed by Gibco BRL (Gaithersburg MD). When sequencing or verifying the sequence of oligonucleotides (such as oligonucleotide made *de novo* by chemical synthesis), the method of Maxam and Gilbert may be preferred (Maxam and Gilbert, 1980, *Meth. Enz.* 65:499; Ausubel et al., *supra*, Ch. 7).

The 5' untranslated sequences of hTRT or other TRT mRNAs can be determined directly by cloning a "full-length" hTRT or other cDNA using standard methods such as reverse transcription of mRNA, followed by cloning and sequencing the resulting cDNA. Preferred oligo(dT)-primed libraries for screening or amplifying full length cDNAs that have been size-selected to include larger cDNAs may be preferred. Random primed libraries are also suitable and often include a larger proportion of clones that contain the 5' regions of genes. Other well known methods for obtaining 5' RNA sequences, such as the RACE protocol described by Frohman et al., 1988, *Proc. Nat. Acad. Sci USA* 85:8998, may also be used. If desired, the transcription start site of an hTRT or other TRT mRNA can be determined by routine methods using the nucleic acids provided herein (e.g., having a sequence of SEQUENCE ID NO: 1). One method is S1 nuclease analysis (Ausubel et al., *supra*) using a labeled DNA having a sequence from the 5' region of SEQUENCE ID NO: 1.

2) CHEMICAL SYNTHESIS OF NUCLEIC ACIDS

The present invention also provides hTRT polynucleotides (RNA, DNA or modified) that are produced by direct chemical synthesis. Chemical synthesis is generally preferred for the production of oligonucleotides or for oligonucleotides and polynucleotides containing nonstandard nucleotides (e.g., probes, primers and antisense oligonucleotides). Direct chemical synthesis of nucleic acids can be accomplished by methods known in the art, such as the phosphotriester method of Narang et al., 1979, *Meth. Enzymol.* 68:90; the phosphodiester method of Brown et al., *Meth. Enzymol.*

68:109 (1979); the diethylphosphoramidite method of Beaucage et al., *Tetra. Lett.*,
22:1859 (1981); and the solid support method of U.S. Patent No. 4,458,066. Chemical
synthesis typically produces a single stranded oligonucleotide, which may be converted
into double stranded DNA by hybridization with a complementary sequence, or by
5 polymerization with a DNA polymerase and an oligonucleotide primer using the single
strand as a template. One of skill will recognize that while chemical synthesis of DNA
is often limited to sequences of about 100 or 150 bases, longer sequences may be
obtained by the ligation of shorter sequences or by more elaborate synthetic methods.

It will be appreciated that the hTRT (or hTR or other) polynucleotides
10 and oligonucleotides of the invention can be made using nonstandard bases (e.g., other
than adenine, cytidine, guanine, thymine, and uridine) or nonstandard backbone
structures to provides desirable properties (e.g., increased nuclease-resistance,
tighter-binding, stability or a desired T_m). Techniques for rendering oligonucleotides
nuclease-resistant include those described in PCT publication WO 94/12633. A wide
15 variety of useful modified oligonucleotides may be produced, including
oligonucleotides having a peptide-nucleic acid (PNA) backbone (Nielsen et al., 1991,
Science 254:1497) or incorporating 2'-O-methyl ribonucleotides, phosphorothioate
nucleotides, methyl phosphonate nucleotides, phosphotriester nucleotides,
phosphorothioate nucleotides, phosphoramidates. Still other useful oligonucleotides
20 may contain alkyl and halogen-substituted sugar moieties comprising one of the
following at the 2' position: OH, SH, SCH₃, F, OCN, OCH₃OCH₃, OCH₃O(CH₂)_nCH₃,
O(CH₂)_nNH₂ or O(CH₂)_nCH₃ where n is from 1 to about 10; C₁ to C₁₀ lower alkyl,
substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF₃; OCF₃; O-, S-, or N-alkyl;
O-, S-, or N-alkenyl; SOCH₃; SO₂CH₃; ONO₂; NO₂; N₃; NH₂; heterocycloalkyl;
25 heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA
cleaving group; a cholesteryl group; a folate group; a reporter group; an intercalator; a
group for improving the pharmacokinetic properties of an oligonucleotide; or a group
for improving the pharmacodynamic properties of an oligonucleotide and other
substituents having similar properties. Folate, cholesterol or other groups which
30 facilitate oligonucleotide uptake, such as lipid analogs, may be conjugated directly or
via a linker at the 2' position of any nucleoside or at the 3' or 5' position of the 3'-

terminal or 5'-terminal nucleoside, respectively. One or more such conjugates may be used. Oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group. Other embodiments may include at least one modified base form or "universal base" such as inosine, or inclusion of other nonstandard bases such as queosine and wybutosine as well as acetyl-, methyl-, thio- and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine which are not as easily recognized by endogenous endonucleases. The invention further provides oligonucleotides having backbone analogues such as phosphodiester, phosphorothioate, phosphorodithioate, methylphosphonate, phosphoramidate, alkyl phosphotriester, sulfamate, 3'-thioacetal, methylene(methylimino), 3'-N-carbamate, morpholino carbamate, chiral-methyl phosphonates, nucleotides with short chain alkyl or cycloalkyl intersugar linkages, short chain heteroatomic or heterocyclic intersugar ("backbone") linkages, or $\text{CH}_2\text{-NH-O-CH}_2$, $\text{CH}_2\text{-N(CH}_3\text{)-OCH}_2$, $\text{CH}_2\text{-O-N(CH}_3\text{)-CH}_2$, $\text{CH}_2\text{-N(CH}_3\text{)-N(CH}_3\text{)-CH}_2$ and $\text{O-N(CH}_3\text{)-CH}_2\text{-CH}_2$ backbones (where phosphodiester is O-P-O-CH_2), or mixtures of the same. Also useful are oligonucleotides having morpholino backbone structures (U.S. Patent No. 5,034,506).

Useful references include Oligonucleotides and Analogues, A Practical Approach, edited by F. Eckstein, IRL Press at Oxford University Press (1991); Antisense Strategies, Annals of the New York Academy of Sciences, Volume 600, Eds. Baserga and Denhardt (NYAS 1992); Milligan et al., 9 July 1993, J. Med. Chem. 36(14):1923-1937; Antisense Research and Applications (1993, CRC Press), in its entirety and specifically Chapter 15, by Sanghvi, entitled "Heterocyclic base modifications in nucleic acids and their applications in antisense oligonucleotides." Antisense Therapeutics, ed. Sudhir Agrawal (Humana Press, Totowa, New Jersey, 1996).

D) LABELING NUCLEIC ACIDS

It is often useful to label the nucleic acids of the invention, for example, when the hTERT or other oligonucleotides or polynucleotides are to be used as nucleic acid probes. The labels (see *infra*) may be incorporated by any of a number of means well known to those of skill in the art. In one embodiment, an unamplified nucleic acid (e.g., mRNA, polyA mRNA, cDNA) is labeled. Means of producing labeled nucleic acids are well known to those of skill in the art and include, for example, nick-translation, random primer labeling, end-labeling (e.g. using a kinase), and chemical conjugation (e.g., photobiotinylation) or synthesis. In another embodiment, the label is simultaneously incorporated during an amplification step in the preparation of the sample nucleic acids. Thus, for example, polymerase chain reaction (PCR) or other nucleic acid amplification method with labeled primers or labeled nucleotides will provide a labeled amplification product. In another embodiment, transcription amplification using a labeled nucleotide (e.g. fluorescein-labeled UTP and/or CTP) incorporates a label into the transcribed nucleic acids. An amplification product may also, or alternatively, be labeled after the amplification is completed.

E) ILLUSTRATIVE OLIGONUCLEOTIDES

As noted *supra* and discussed in detail *infra*, oligonucleotides are used for a variety of uses including as primers, probes, therapeutic or other antisense oligonucleotides, triplex oligonucleotides, and numerous other uses as apparent from this disclosure. Table 2 provides certain illustrative specific oligonucleotides that may be used in the practice of the invention. It will be appreciated that numerous other useful oligonucleotides of the invention may be synthesized by one of skill, following the guidance provided herein.

In Table 2, "seq" means that the primer has been used, or is useful, for sequencing; "PCR" means that the primer has been used, or is useful, for PCR; "AS" means that means that the primer has been used, or is useful for antisense inhibition of telomerase activity; "CL" means that the primer has been used, or is useful in cloning regions of hTERT genes or RNA, "mut" means that the primer has been used, or is useful for constructing mutants of hTERT genes or gene products. "UC" means "upper

case," and "lc" means "lower case." Mismatches and insertions (relative to SEQUENCE ID NO: 1) are indicated by underlining; deletions are indicated by a "-".

It will be appreciated that nothing in Table 2 is intended to limit the use of any particular oligonucleotide to any single use or set of uses.

TABLE 2
USEFUL OLIGONUCLEOTIDES

| primer | 3'-sequence-5' | Notes | unimatch2.2 | USE | | | | |
|----------|-------------------------|-------|-------------|-----|-----|----|----|-----|
| | | | | seq | PCR | AS | CL | MUT |
| TCP1.1 | GTGAAGGCACCTGTTACGG | | | x | x | | | |
| TCP1.2 | GTGGATGATTTCTTGTTGG | | | x | x | | | |
| TCP1.4 | CTGGACACTCAGCCCTTGG | | | x | x | | | |
| TCP1.5 | GGCAGGATGCTGACACT | | | x | x | | | |
| TCP1.6 | TTTGAATGATGCTGGCATG | | | x | x | | | |
| TCP1.7 | GGGGCTGCTCTTCTACAGG | | | x | x | | | |
| TCP1.8 | CAGCAGGAGGATCTTGTAG | | | x | x | | | |
| TCP1.9 | TGACCCAGGAGTGGCAGG | | | x | x | | | |
| TCP1.10 | TCAAGCTGACTCGACACCG | | | x | x | | | |
| TCP1.11 | CGGCGTGACAGGGCTGC | | | x | x | | | |
| TCP1.12 | GCTGAAGGCTGAGTGTCC | | | x | x | | | |
| TCP1.13 | TAGTCCATGTTCACAATCG | | | x | x | | | |
| TCP1.14 | CTGTGCTGGGCTGGACUAT | | | x | x | | | |
| TCP1.15 | CGCGTGGGTGAGGTGAGGTG | | | x | x | | | |
| TCP1.16 | TTTCCGTGTTGAGTGTTC | | | x | x | | | |
| TCP1.17 | GTCACCGTGTGGGCAAG | | | x | x | | | |
| TCP1.19 | GCTACCTGCCCAACACCG | | | x | x | | | |
| TCP1.20 | GCGCAAGAACGTGCTCG | | | x | x | | | |
| TCP1.21 | CA-CTGCTCCTTGTGCGCTG | | | x | x | | | |
| TCP1.22 | TTCCCAAGGACTTGTGTTC | | | x | x | | | |
| TCP1.24 | TGTTCTCAAGACGCACTG | | | x | x | | | |
| TCP1.25 | TACTGGGTGCGTGGTATG | | | x | x | | | |
| TCP1.26 | GGTCTTGGGGCTGAAGTGT | | | x | x | | | |
| TCP1.27 | TGGTTCACCTGCTGGCAG | | | x | x | | | |
| TCP1.28 | GTGGTTCTGTGTGTGTC | | | x | x | | | |
| TCP1.29 | GACACACACAGAAACAC | | | x | x | | | |
| TCP1.30 | GTGCCAGCAGGTGAACAG | | | x | x | | | |
| TCP1.32B | GCAGTGCGTCTTGAGGAGC | | | x | x | | | |
| TCP1.33 | TGGAAACATAGCCTCAAGGAG | | | x | x | | | |
| TCP1.34 | GCCCTCCCTGACGCTATGCTT | | | x | x | | | |
| TCP1.35 | GC(GT)CGGCGCTGCCACTCAGG | | | x | x | | | |
| TCP1.35i | GCTCGGCGCTGCCACTCAGG | | | x | x | | | |
| TCP1.36 | ACCCGAGACCAAGCACTTC | | | x | x | | | |

TABLE 2
(cont.)

| | | | |
|---------|-----------------------------|---|---|
| TCP1.38 | CCAAAGAGGTGGCTTCTTCG | x | x |
| TCP1.39 | AAGCCAGCACGTTCTTCGC | x | x |
| TCP1.40 | CACGTTGTCGGCGGCTTG | x | x |
| TCP1.41 | CCTTCAACCACCAAGCGTGG | x | x |
| TCP1.42 | GGGACGACGTTGCTGTTTC | x | x |
| TCP1.43 | GGCTCAGGGGCAAGCGCCAC | x | x |
| TCP1.44 | CTGGCAAGTGTACGGCTTC | x | x |
| TCP1.45 | GGCTGGACCGAGTGAACCTGTGTTTC | x | x |
| TCP1.46 | GACGTGTTGGCCGCAITGTTG | x | x |
| TCP1.47 | GAACTGTGCGGTTGCTCAAGAG | x | x |
| TCP1.48 | GACACCAACAGAAACCAAGTTCAT | x | x |
| TCP1.49 | CGCCCTCTCTCCGCAAGT | x | x |
| TCP1.50 | CGAAGCCGAAGGCCAATACGTTCTT | x | x |
| TCP1.51 | GATGGCCCAAGTCTTCAGAGG | x | x |
| TCP1.52 | GTAGCTGGCCAGGCTGCTGTGTAAG | x | x |
| TCP1.53 | TGGCGAAGCAAGTCTGTGTTCA | x | x |
| TCP1.54 | TATGTTCCAGGCCGTTCCGATTC | x | x |
| TCP1.55 | CCAGCTGGCCCTACCAAGTGTGC | x | x |
| TCP1.56 | GGCTCCCTGACGGCTATGTTCCAG | x | x |
| TCP1.57 | GGTGTGCCGCTGGCCACGTTCTG | x | x |
| TCP1.58 | TCCCAAGGGCAAGCAACCAAGGCACT | x | x |
| TCP1.59 | GTACAAGGGCAACCTTTGTTCACTTC | x | x |
| TCP1.60 | TGACGAGGTACACACTTCATCAGCC | x | x |
| TCP1.61 | AGCGGCAAGCACTCGCGGTAGTGGC | x | x |
| TCP1.62 | CCACCAAGTCTCTCAAGCAAGACAC | x | x |
| TCP1.63 | CCAGGGCTTCCACAGTGGCGCAAGCAG | x | x |
| TCP1.64 | CGCACGAMCGTGGCCAGCGGCAACA | x | x |
| TCP1.65 | TGACCGTGGTTTCTGTGTTGTT | x | x |
| TCP1.66 | CCCTCTTCAAGTGTGTTCTGATTCC | x | x |
| TCP1.67 | ATCGCGGCCACCAAGTCCCT | x | x |
| TCP1.68 | TGCTCAGACACTCGGCGGTAGAA | x | x |
| TCP1.69 | ACGAAGCCGTACACCTGGC | x | x |
| TCP1.72 | CGACATCCCTGCTTCTTGGCTTTTC | x | x |
| TCP1.73 | CACGTGCTGGCTCATTTCAAGG | x | x |
| TCP1.74 | GCGACATGGAGAACAAAGC | x | x |
| TCP1.75 | GCAGCCATACTCAGGGACAC | x | x |
| TCP1.76 | CCAATCTCTCCACGCTGCTC | x | x |

(cont.)

42

TABLE 2
(cont.)

| | | | | | |
|---------|---|--|---|---|---|
| PE02 | CCAGGGCTTCCACAGTCCGACGACGACGCGC | Xlin I site / IIA tag / hTRT | x | x | |
| LM101 | CTAGCTAGATCA / GCTAGCGTAATCTGGAMCATCGTA TGGGTA / GTCCAGGATGGCTCTGAAGTC | into pGRN121 | | | x |
| LM103 | TACCATGGGCTACCCCATACGACCGTTCCAGATTACGCTCA | inserts IIA tag into a Nde I site at 5' end of hTRT | | | x |
| LM104 | TATGAGCGTAATCTGGAACGTCTGATGGGTAGCCCATGG | anneals to LM103 | | | x |
| LM105 | GTGTACGTCGTGAGCTCTCTCAGGTCTGCTTTT | change = F539A (phc > ala) | | | x |
| LM106 | ATGTACACGAG | change = F360A (phc > ala) | | | x |
| LM107 | GTGTACGTCGTGAGCTCTCTCAGGTCTCTGCTTTATGTC | change = Y361A (tyr > ala) | | | x |
| LM108 | ACGAGAGACC CCTCAGGCTCTTTCTTTGCTGTACGAGACAAACGTTT CAAAAGAACAG GGCTCTTTTATGTGCGGAGACAAACGTTT | change = T363A (thr > ala) | | | x |
| LM109 | CTTCTTTTATGTACACGGGACAAACGTTTCAAAAGAACAA | change = E564A deletion of FFYVTE (aa 559-564) | | | x |
| LM_FFYT | ATGAGTGTGTACGTCGTGAGCTCTCTCAGGTCTACACAG | complement to TCP1.61 to DD motif, designed to possibly anneal to mTRT | x | x | x |
| TCP061: | CAAAAGAACAGGCTCTTTTTC | to DD motif, designed to possibly anneal to mTRT | x | | |
| HUM01: | GGCTGATGAGTGTGTACGTCGTGAG | designed to possibly anneal to mTRT | x | | |
| HUM01: | ACGTGGTCTCCGTGACATTAAGAA | designed to possibly anneal to mTRT | x | | |
| HUM02: | AGGTCITTTCTTTTATGTACAGGA | designed to possibly anneal to mTRT | x | | |
| HUM03: | CACAGACCCCGCTCGCTGTGTC | designed to possibly anneal to mTRT | x | | |
| HUM04: | CCGAGTCTGGAAGTCAAGCAGGGC | designed to possibly anneal to mTRT | x | | |
| SLW F1N | CGGATCTCTGGAAGTCAAGCAGGGC | possibly anneal to mTRT | x | | |
| SLW F1C | CGGATCTCTGGAAGTCAAGCAGGGC | possibly anneal to mTRT | x | | |
| SLW F2N | CGGATCTCTGGAAGTCAAGCAGGGC | possibly anneal to mTRT | x | | |
| SLW F2C | CGGATCTCTGGAAGTCAAGCAGGGC | possibly anneal to mTRT | x | | |
| SLW F3N | CGGATCTCTGGAAGTCAAGCAGGGC | possibly anneal to mTRT | x | | |

44

| SLW F3C | SLW F3N / SLW F3C amplify a 887 nt piece of pGIRN121 (2426 to 3274) | SLW F4C | SLW F4N / SLW F4C amplify a 944 nt piece of pGIRN121 (3272 to 4177) |
|---|--|--|--|
| <p> GGCAATCGGAGGATCCCTGGCACATGGACG 260-280 500-520 770-790 885-905 1000-1020 1300-1320 1520-1540 2110-2130 2295-2315 2450-2470 2670-2690 3080-3110 3140-3160 3690-3710 55-75 151-171 171-1 171-2 171-3 171-4 </p> | <p> GGCAATCGGAGGATCCCTGGCACATGGACG GGCAACCTGCGCGGAGGAGAGG GCGTGCACGACGGTGAACCA CTCAGGGGCGACGCCACGCT AGCTGGCTCTTGGCGGCTG GGACAMGGCGTGTCCAGGGA GCTGGGGTGACCGGAGCTGG GATGAACCTCTTGCTGCTCT GTGGGCGACGCCCTGTGGAT GCCCATGGCGGCGCTCTGGA GAGGCCACTGCTGGCCCTAT GGGTGAGGTGAGGTGTACCA GCTGCACACACATGCTGAACCTGTACCG GACGGCGAGGAAAAATGTGG CCGAGCGGCAAGCTGTGGGA CAGCGGGGAGCGCGGGGCA CAGCACTCGCGGTAGTGGCT TCAGGCCAAACCTGAATCTGAG CCCGAGTGAATCTTCTACGC GTCTGGGAGTTCTCTCATCC TTTAGGCATCTCTCCCAAGCACA </p> | <p> GGCAATCGGAGGATCCCTGGCACATGGACG GGCAACCTGCGCGGAGGAGAGG GCGTGCACGACGGTGAACCA CTCAGGGGCGACGCCACGCT AGCTGGCTCTTGGCGGCTG GGACAMGGCGTGTCCAGGGA GCTGGGGTGACCGGAGCTGG GATGAACCTCTTGCTGCTCT GTGGGCGACGCCCTGTGGAT GCCCATGGCGGCGCTCTGGA GAGGCCACTGCTGGCCCTAT GGGTGAGGTGAGGTGTACCA GCTGCACACACATGCTGAACCTGTACCG GACGGCGAGGAAAAATGTGG CCGAGCGGCAAGCTGTGGGA CAGCGGGGAGCGCGGGGCA CAGCACTCGCGGTAGTGGCT TCAGGCCAAACCTGAATCTGAG CCCGAGTGAATCTTCTACGC GTCTGGGAGTTCTCTCATCC TTTAGGCATCTCTCCCAAGCACA </p> | <p> GGCAATCGGAGGATCCCTGGCACATGGACG GGCAACCTGCGCGGAGGAGAGG GCGTGCACGACGGTGAACCA CTCAGGGGCGACGCCACGCT AGCTGGCTCTTGGCGGCTG GGACAMGGCGTGTCCAGGGA GCTGGGGTGACCGGAGCTGG GATGAACCTCTTGCTGCTCT GTGGGCGACGCCCTGTGGAT GCCCATGGCGGCGCTCTGGA GAGGCCACTGCTGGCCCTAT GGGTGAGGTGAGGTGTACCA GCTGCACACACATGCTGAACCTGTACCG GACGGCGAGGAAAAATGTGG CCGAGCGGCAAGCTGTGGGA CAGCGGGGAGCGCGGGGCA CAGCACTCGCGGTAGTGGCT TCAGGCCAAACCTGAATCTGAG CCCGAGTGAATCTTCTACGC GTCTGGGAGTTCTCTCATCC TTTAGGCATCTCTCCCAAGCACA </p> |

IV. TRT PROTEINS AND PEPTIDES

A) GENERALLY

The invention provides a wide variety of hTERT proteins useful for, *inter alia*, production of telomerase activity, inhibition of telomerase activity in a cell, induction of an anti-hTERT immune response, as a therapeutic reagent, as a standard or control in a diagnostic assay, as a target in a screen for compounds capable of activation or inhibition of an activity of hTERT or telomerase, and numerous other uses that will be apparent to one of skill or are otherwise described herein. The hTERT of the invention include functionally active proteins (useful for e.g., conferring telomerase activity in a telomerase-negative cell) and variants, inactive variants (useful for e.g., inhibiting telomerase activity in a cell), hTERT polypeptides, and telomerase RNPs (e.g., ribonucleoprotein complexes comprising the proteins) that exhibit one, several, or all of the functional activities of naturally occurring hTERT and telomerase, as discussed in greater detail for illustrative purposes, below.

In one embodiment, the hTERT protein of the invention is a polypeptide having a sequence as set forth in Figure 17 (SEQUENCE ID NO: 2), or a fragment thereof. In another embodiment, the hTERT polypeptide differs from SEQUENCE ID NO: 2 by internal deletions, insertions, or conservative substitutions of amino acid residues. In a related embodiment, the invention provides hTERT polypeptides with substantial similarity to SEQUENCE ID NO: 2. The invention further provides hTERT polypeptides that are modified, relative to the amino acid sequence of SEQUENCE ID NO: 2, in some manner, e.g., truncated, mutated, derivatized, or fused to other sequences (e.g., to form a fusion protein). Moreover, the present invention provides telomerase RNPs comprising an hTERT protein of the invention complexed with a template RNA (e.g., hTR). In other embodiments, one or more telomerase-associated proteins is associated with hTERT protein and/or hTR.

The invention also provides other naturally occurring hTERT species or nonnaturally occurring variants, such as proteins having the sequence of, or substantial similarity to SEQUENCE ID NO: 5 [Figure 20], SEQUENCE ID NO: 10 [Figure 19], and fragments, variants, or derivatives thereof.

The invention provides still other hTERT species and variants. One

example of an hTRT variant may result from ribosome frameshifting of mRNA encoded by the clone 712562 (SEQUENCE ID NO: 3 [Figure 18]) or the pro90 variant hTRT shown in SEQUENCE ID NO: 4 [Figure 20] and so result in the synthesis of hTRT polypeptides containing all the TRT motifs (for a general example, see, e.g.,
5 Tsuchihashi et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:2516; Craigengen et al., 1987, *Cell* 50:1; Weiss, 1990, *Cell* 62:117). Ribosome frameshifting can occur when specific mRNA sequences or secondary structures cause the ribosome to "stall" and jump one nucleotide forwards or back in the sequence. Thus, a ribosome frameshift event on the 712562 mRNA could cause the synthesis of an approximately 523 amino acid residue
10 polypeptide. A ribosome frameshift event on the pro90 sequence could result in a protein with approximately 1071 residues. It will be appreciated that proteins resulting from ribosome frameshifting can also be expressed by synthetic or recombinant techniques provided by the invention.

Human TRT proteins, peptides, and functionally equivalent proteins
15 may be obtained by purification, chemical synthesis, or recombinant production, as discussed in greater detail below.

B) TRT PROTEIN ACTIVITIES

The TRT polypeptides of the invention (including fragments, variants,
20 products of alternative alleles, and fusion proteins) can have one or more, or all of the functional activities associated with native hTRT. Except as noted, as used herein, an hTRT or other TRT polypeptide is considered to have a specified activity if the activity is exhibited by either the hTRT protein without an associated RNA (e.g., hTR) or in an hTRT-associated RNA (e.g., hTR) complex. The hTR-binding activity of hTRT is one
25 example of an activity associated with the hTRT protein. Methods for producing complexes of nucleic acids (e.g., hTR) and the hTRT polypeptides of the invention are described *infra*.

Modification of the hTRT protein (e.g., by chemical or recombinant means, including mutation or modification of a polynucleotide encoding the hTRT
30 polypeptide or chemical synthesis of a polynucleotide that has a sequence different than a native polynucleotide sequence) to have a different complement of activities than

native hTERT can be useful in therapeutic applications or in screening for specific modulators of hTERT or telomerase activity. In addition, assays for various hTERT activities can be particularly useful for identification of agents (e.g., activity modulating agents) that interact with hTERT or telomerase to change telomerase activity.

5 The activities of native hTERT, as discussed *infra*, include telomerase catalytic activity (which may be either processive or non-processive activity); telomerase processivity; conventional reverse transcriptase activity; nucleolytic activity; primer or substrate (telomere or synthetic telomerase substrate or primer) binding activity; dNTP binding activity; RNA (i.e., hTR) binding activity; and protein
10 binding activity (e.g., binding to telomerase-associated proteins, telomere-binding proteins, or to a protein-telomeric DNA complex). It will be understood, however, that present invention also provides hTERT compositions without any particular hTERT activity but with some useful activity related to the hTERT or other TERT proteins (e.g., certain typically short immunogenic peptides, inhibitory peptides).

15

1) TELOMERASE CATALYTIC ACTIVITY

As used herein, a polypeptide of the invention has "telomerase catalytic activity," when the polypeptide is capable of extending a DNA primer that functions as a telomerase substrate by adding a partial, one, or more than one repeat of a sequence
20 (e.g., TTAGGG) encoded by a template nucleic acid (e.g., hTR). This activity may be processive or nonprocessive. Processive activity occurs when a telomerase RNP adds multiple repeats to a primer or telomerase before the DNA is released by the enzyme complex. Non-processive activity occurs when telomerase adds a partial, or only one, repeat to a primer and is then released. *In vivo*, however, a non-processive reaction
25 could add multiple repeats by successive rounds of association, extension, and dissociation. This can occur *in vitro* as well, but it is not typically observed in standard assays due to the vastly large molar excess of primer over telomerase in standard assay conditions.

To characterize an hTERT polypeptide as having non-processive activity, a
30 conventional telomerase reaction is performed using conditions that favor a non-processive reaction, for example high temperatures (i.e., 35-40°C, typically 37°C).

low dGTP concentrations (1 μ M or less), high primer concentrations (5 μ M or higher), and high dATP/TTP concentrations (2 mM or higher), with the temperature and dGTP typically having the greatest effect. To characterize an hTRT polypeptide as having processive activity, a conventional telomerase reaction is performed using conditions that favor a processive reaction (for example, 27-34°C, typically 30°C), high dGTP concentration (10 μ M or higher), low primer concentration (1 μ M or lower), and/or low dATP and TTP concentrations (0.3-1 mM) with temperature and dGTP typically concentration being the most critical. Alternatively, a TRAP assay (for processive or moderately processive activity) or the dot-blot and gel blot assays (for processive activity) may be used. The hTRT polypeptide of the invention can possess a non-processive activity, but not a processive activity (e.g., if an alteration of the hTRT polypeptide reduces or eliminates the ability to translocate), can be solely processive, or can possess both activities.

a) Non-processive Activity

A non-processive telomerase catalytic activity can extend the DNA primer from the position where the 3' end anneals to the RNA template to the 5' end of the template sequence, typically terminating with the addition of the first G residue (as, for example, when the template is hTR). As shown in Table 3, the exact number of nucleotides added is dependent on the position of the 3' terminal nucleotide of the primer in the TTAGGG repeat sequence.

TABLE 3
NONPROCESSIVE ACTIVITY

i). -----TTAGGGttag (DNA)
3'-----AUCCCAAUC-----5' (RNA)

ii) -----TTAGggttag (DNA)
3'-----AUCCCAAUC-----5' (RNA)

In DNA, UC = primer, lc = added nucleotides

Thus, 4 nucleotides are added to the --TTAGGG primer (i) while 6 nucleotides are added to the --TTAG primer (ii). The first repeat added by telomerase in a processive reaction is equivalent to this step; however, in a processive reaction telomerase performs a translocation step where the 3' end is released and re-bound at

the 3' region of the template in a position sufficient to prime addition of another repeat (see Morin, 1997, *Eur. J. Cancer* 33:750).

A fully non-processive reaction produces only one band in a conventional assay using a single synthetic primer. Because this result could also be produced by other enzymes, such as a terminal transferase activity, it may be desirable in some applications to verify that the product is a result of a telomerase catalytic activity. A telomerase (comprising hTERT) generated band can be distinguished by several additional characteristics. The number of nucleotides added to the end of the primer should be consistent with the position of the primer 3' end. Thus, a --TTAGGG primer should have 4 nucleotides added and a --TTAG primer should have 6 nucleotides added (see above). In practice, two or more sequence permuted primers can be used which have the same overall length but different 5' and 3' endpoints. As an illustrative example, the non-processive extension of primers 5'-TTAGGGTTAGGGTTAGGG and 5'-GTTAGGGTTAGGGTTAGG will generate products whose absolute length will be one nucleotide different (4 added to 5'-TTAGGGTTAGGGTTAGGG for a 22 nt total length, and 5 added to 5'-GTTAGGGTTAGGGTTAGG for a 23 nt total length). The nucleotide dependence of the reaction should be consistent with the position of the primer terminus. Thus, a --TTAGGG primer product should require dGTP, TTP, and dATP, but not dCTP, and a ---AGGGTT primer product should require dGTP and dATP, but not TTP or dCTP. The activity should be sensitive to RNAase or micrococcal nuclease pre-treatment (see Morin, 1989, *Cell* 59: 521) under conditions that will degrade hTR and so eliminate the template.

b) Processive Activity

In practice, a processive activity is easily observed by the appearance of a six nucleotide ladder in a conventional assay, TRAP assay, or gel-blot assay. A dot-blot assay can also be used, but no ladder is detected in such a method. The conventional assay is described in Morin, 1989, *Cell* 59:521, which is incorporated herein in its entirety and for all purposes. The TRAP assay is described in U.S. Patent No. 5,629,154; see also, PCT publication WO 97/15687, PCT publication WO

95/13381; Krupp et al. *Nucleic Acids Res.*, 1997, 25: 919; and Wright et al., 1995, *Nuc. Acids Res.* 23:3794, each of which is incorporated herein in its entirety and for all purposes. The dot blot assay can be used in a format in which a non-processive activity, which does not add the 3 or more repeats required for stable hybridization of the (CCC_nUAA)_n probe used to detect the activity, is tested with compounds or hTERT variants to determine if the same generates processivity, i.e., if the probe detects an expected telomerase substrate, then the compound or mutant is able to change the non-processive activity to a processive activity. Other assays for processive telomerase catalytic activity can also be used, e.g., the stretch PCR assay of Tatematsu et al., 1996, *Oncogene* 13:2265. The gel-blot assay, a combination of the conventional and dot blot assays can also be used. In this variation a conventional assay is performed with no radiolabeled nucleotide and with high dGTP concentrations (e.g., 0.1-2 mM). After performing the conventional assay, the synthesized DNA is separated by denaturing PAGE and transferred to a membrane (e.g., nitrocellulose). Telomeric DNA (the product of telomerase - an extended telomerase primer or substrate) can then be detected by methods such as hybridization using labeled telomeric DNA probes (e.g., probes containing the CCCTAA sequence, as used in the dot blot assay, *supra*). An advantage of this technique is that it is more sensitive than the conventional assay and provides information about the size of the synthesized fragments and processivity of the reaction.

c) Activity determinations

The telomerase activity of an hTERT polypeptide can be determined using an unpurified, partially purified or substantially purified hTERT polypeptide (e.g., in association with hTR), *in vitro*, or after expression *in vivo*. For example, telomerase activity in a cell (e.g., a cell expressing a recombinant hTERT polypeptide of the invention) can be assayed by detecting an increase or decrease in the length of telomeres. Typically assays for telomerase catalytic activity are carried out using an hTERT complexed with hTR; however, alternative telomerase template RNAs may be substituted, or one may conduct assays to measure another activity, such as telomerase-primer binding. Assays to determine the length of telomeres are known in the art and

include hybridization of probes to telomeric DNA (an amplification step can be included) and TRF analysis i.e., the analysis of telomeric DNA restriction fragments [TRFs] following restriction endonuclease digestion, see PCT publications WO 93/23572 and WO 96/41016; Counter et al., 1992, *EMBO J.* 11:1921; Allsopp et al., 5 1992, *Proc. Nat'l. Acad. Sci. USA* 89:10114; Sanno, 1996, *Am J Clin Pathol* 106:16 and Sanno, 1997, *Neuroendocrinology* 65:299.

The telomerase catalytic activity of an hTERT polypeptide may be determined in a number of ways using the assays *supra* and other telomerase catalytic activity assays. According to one method, the hTERT protein is expressed (e.g., as 10 described *infra*) in a telomerase negative human cell in which hTR is expressed (i.e., either normally in the cell or through recombinant expression), and the presence or absence of telomerase activity in the cell or cell lysate is determined. Examples of suitable telomerase-negative cells are IMR 90 (ATCC, #CCL-186) or BJ cells (human foreskin fibroblast line; see, e.g., Feng et al., 1995, *Science* 269:1236). Other 15 examples include retinal pigmented epithelial cells (RPE), human umbilical vein endothelial cells (HUVEC: ATCC #CRL-1730), human aortic endothelial cells (HAEC: Clonetics Corp. #CC-2535), and human mammary epithelial cells (HME: Hammond et al., 1984, *Proc. Nat'l. Acad. Sci. USA* 81:5435; Stampfer, 1985, *J. Tissue Culture Methods* 9:107). In an alternative embodiment, the hTERT polypeptide is expressed 20 (e.g., by transfection with an hTERT expression vector) in a telomerase positive cell, and an increase in telomerase activity in the cell compared to an untransfected control cell is detected if the polypeptide has telomerase catalytic activity. Usually the telomerase catalytic activity in a cell transfected with a suitable expression vector expressing hTERT will be significantly increased, such as at least about 2-fold, at least about 5-fold, or 25 even at least about 10-fold to 100-fold or even 1000-fold higher than in untransfected (control) cells.

In an alternative embodiment, the hTERT protein is expressed in a cell (e.g., a telomerase negative cell in which hTR is expressed) as a fusion protein (see 30 *infra*) having a label or an "epitope tag" to aid in purification. In one embodiment, the RNP is recovered from the cell using an antibody that specifically recognizes the tag. Preferred tags are typically short or small and may include a cleavage site or other

property that allows the tag to be removed from the hTRT polypeptide. Examples of suitable tags include the Xpress™ epitope (Invitrogen, Inc., San Diego CA), and other moieties that can be specifically bound by an antibody or nucleic acid or other equivalent method such as those described in Example 6. Alternative tags include those encoded by sequences inserted, e.g., into SEQUENCE ID NO: 1 upstream of the ATG codon that initiates translation of the protein of SEQUENCE ID NO: 2, which may include insertion of a (new) methionine initiation codon into the upstream sequence.

It will be appreciated that when an hTRT variant is expressed in a cell (e.g., as a fusion protein) and subsequently isolated (e.g., as a ribonucleoprotein complex), other cell proteins (i.e., telomerase-associated proteins) may be associated with (directly or indirectly bound to) the isolated complex. In such cases, it will sometimes be desirable to assay telomerase activity for the complex containing hTRT, hTR and the associated proteins.

2) OTHER TELOMERASE OR TRT PROTEIN ACTIVITIES

The hTRT polypeptides of the invention include variants that lack telomerase catalytic activity but retain one or more other activities of telomerase. These other activities and the methods of the invention for measuring such activities include (but are not limited to) those discussed in the following sections.

a) Conventional reverse transcriptase activity

Telomerase conventional reverse transcriptase activity is described in, e.g., Morin, 1997, *supra*, and Spence et al., 1995, *Science* 267:988. Because hTRT contains conserved amino acid motifs that are required for reverse transcriptase catalytic activity, hTRT has the ability to transcribe certain exogenous (e.g., non-hTR) RNAs. A conventional RT assay measures the ability of the enzyme to transcribe an RNA template by extending an annealed DNA primer. Reverse transcriptase activity can be measured in numerous ways known in the art, for example, by monitoring the size increase of a labeled nucleic acid primer (e.g., RNA or DNA), or incorporation of a labeled dNTP. See, e.g., Ausubel et al., *supra*.

Because hTRT specifically associates with hTR, it can be appreciated

that the DNA primer/RNA template for a conventional RT assay can be modified to have characteristics related to hTR and/or a telomeric DNA primer. For example, the RNA can have the sequence (CCCTAA)_n, where n is at least 1, or at least 3, or at least 10 or more. In one embodiment, the (CCCTAA)_n region is at or near the 5' terminus of the RNA (similar to the 5' locations of template regions in telomerase RNAs). Similarly, the DNA primer may have a 3' terminus that contains portions of the TTAGGG telomere sequence, for example X_nTTAG, X_nAGGG, X_n(TTAGGG)_qTTAG, etc., where X is a non-telomeric sequence and n is 8-20, or 6-30, and q is 1-4. In another embodiment, the DNA primer has a 5' terminus that is non-complementary to the RNA template, such that when the primer is annealed to the RNA, the 5' terminus of the primer remains unbound. Additional modifications of standard reverse transcription assays that may be applied to the methods of the invention are known in the art.

b) Nucleolytic activity

Telomerase nucleolytic activity is described in e.g., Morin, 1997, *supra*; Collins and Grieder, 1993, *Genes and Development* 7:1364. Telomerase possesses a nucleolytic activity (Joyce and Steitz, 1987, *Trends Biochem. Sci.* 12:288); however, telomerase activity has defining characteristics. Telomerase preferentially removes nucleotides, usually only one, from the 3' end of an oligonucleotide when the 3' end of the DNA is positioned at the 5' boundary of the DNA template sequence. In humans and *Tetrahymena*, this nucleotide is the first G of the telomeric repeat (TTAGG in humans). Telomerase preferentially removes G residues but has nucleolytic activity against other nucleotides. This activity can be monitored. Two different methods are described here for illustrative purposes. One method involves a conventional telomerase reaction with a primer that binds the entire template sequence (i.e., terminating at the template boundary; 5'-TAGGGATTAG in humans). Nucleolytic activity is observed by monitoring the replacement of the last dG residue with a radiolabeled dGTP provided in the assay. The replacement is monitored by the appearance of a band at the size of the starting primer as shown by gel electrophoresis and autoradiography.

A preferred method uses a DNA primer that has a "blocked" 3' terminus that cannot be extended by telomerase. The 3'-blocked primer can be used in a standard

telomerase assay but will not be extended unless the 3' nucleotide is removed by the nucleolytic activity of telomerase. The advantage of this method is that telomerase activity can be monitored by any of several standard means, and the signal is strong and easy to quantify. The blocking of the 3' terminus of the primer can be accomplished in several ways. One method is the addition of a 3'-deoxy-dNTP residue at the 3' terminus of the primer using standard oligonucleotide synthesis techniques. This terminus has a 2' OH but not the 3' OH required for telomerase. Other means of blocking the 3' terminus exist, for instance, a 3' dideoxy terminus, a 3'-amine terminus, and others. An example of a primer for an hTERT nucleolytic assay is 5' -TTAGGGTTAGGGTTA (G_{3H}) where the last residue denotes a 3'-deoxy-guanosine residue (Glen Research, Sterling, VA). Numerous other variations for a suitable primer based on the disclosure are known to those of skill in the art.

c) Primer (telomere) binding activity

Telomerase primer (telomere) binding activity is described in e.g., Morin, 1997, *supra*; Collins et al., 1995, *Cell* 81:677; Harrington et al. 1995, *J. Biol. Chem.* 270:8893. Telomerase is believed to have two sites which bind a telomeric DNA primer. The RT motifs associated with primer binding indicate hTERT and/or hTERT/hTR possesses DNA primer binding activity. There are several ways of assaying primer binding activity; however, a step common to most methods is incubation of a labeled DNA primer with hTERT or hTERT/hTR or other TRT/TR combinations under appropriate binding conditions. Also, most methods employ a means of separating unbound DNA from protein-bound DNA; those methods include the following.

i) Gel-shift assays (also called electrophoretic/mobility shift assays) are those in which unbound DNA primer is separated from protein-bound DNA primer by electrophoresis on a nondenaturing gel (Ausubel et al., *supra*).

ii) Matrix binding assays include several variations to the basic technique, which involves binding the hTERT or hTERT/hTR complex to a matrix (e.g., nitrocellulose), either before or after incubation with the labeled primer. By binding the hTERT to a matrix, the unbound primer can be mechanically separated from bound primer. Residual unbound DNA can be removed by washing the membrane prior to quantitation. Those of skill recognize there are several means of coupling proteins to

such matrices, solid supports, and membranes, including chemical, photochemical, UV cross-linking, antibody/epitope, and non-covalent (hydrophobic, electrostatic, etc.) interactions.

The DNA primer can be any DNA with an affinity for telomerase, such as, for example, a telomeric DNA primer like (TTAGGG)_n, where n could be 1-10 and is typically 3-5. The 3' and 5' termini can end in any location of the repeat sequence. The primer can also have 5' or 3' extensions of non-telomeric DNA that could facilitate labeling or detection. The primer can also be derivatized, e.g., to facilitate detection or isolation.

d) dNTP binding activity

Telomerase dNTP binding activity is described in e.g., Morin, 1997, *supra*; Spence et al., *supra*. Telomerase requires dNTPs to synthesize DNA. The hTERT protein has a nucleotide binding activity and can be assayed for dNTP binding in a manner similar to other nucleotide binding proteins (Kantrowitz et al., 1980, *Trends Biochem. Sci.* 5:124). Typically, binding of a labeled dNTP or dNTP analog can be monitored as is known in the art for non-telomerase RT proteins.

e) RNA (i.e., hTR) binding activity

Telomerase RNA (i.e., hTR) binding activity is described in e.g., Morin, 1997, *supra*; Harrington et al., 1997, *Science* 275:973; Collins et al., 1995, *Cell* 81:677. The RNA binding activity of a TRT protein of the invention may be assayed in a manner similar to the DNA primer binding assay described *supra*, using a labeled RNA probe. Methods for separating bound and unbound RNA and for detecting RNA are well known in the art and can be applied to the activity assays of the invention in a manner similar to that described for the DNA primer binding assay. The RNA can be full length hTR, fragments of hTR or other RNAs demonstrated to have an affinity for telomerase or hTERT. See U.S. Patent No. 5,583,016 and PCT Pub. No. 96/40868.

3) TELOMERASE MOTIFS AS TARGETS

The present invention, as noted *supra*, provides in addition to recombinant hTERT with a full complement (as described *supra*) of activities, hTERT polypeptides having less than the full complement of the telomerase activities of

naturally occurring telomerase or hTERT or other TRT proteins. It will be appreciated that, in view of the disclosure herein of the RT and telomerase-specific motifs of TRT, alteration or mutation of conserved amino acid residues, such as are found in the motif sequences discussed *supra*, will result in loss-of activity mutants useful for therapeutic, drug screening and characterization, and other uses. For example, as described in Example 1, deletion of motifs B through D in the RT domains of the endogenous TRT gene in *S. pombe* resulted in haploid cells in which telomere progressively shortened to the point where hybridization of a telomere probe to telomeric repeats became almost undetectable, indicating a loss of telomerase catalytic activity. Similarly, alterations in the WxGxS site of motif E can affect telomerase DNA primer binding or function. Additionally, alterations of the amino acids in the motifs A, B', and C can affect the catalytic activity of telomerase. Mutation of the DD motif of hTERT can significantly reduce or abolish telomerase activity (see Example 16).

C) SYNTHESIS OF HTERT AND OTHER TRT POLYPEPTIDES

The invention provides a variety of methods for making the hTERT and other TRT polypeptides disclosed herein. In the following sections, chemical synthesis and recombinant expression of hTERT proteins, including fusion proteins, is described in some detail.

1) CHEMICAL SYNTHESIS

The invention provides hTERT polypeptides synthesized, entirely or in part, using general chemical methods well known in the art (see e.g., Caruthers et al., 1980, *Nucleic Acids Res. Symp. Ser.*, 215-223; and Horn et al., 1980, *Nucleic Acids Res. Symp. Ser.*, 225-232). For example, peptide synthesis can be performed using various solid-phase techniques (Roberge, et al., 1995, *Science* 269:202), including automated synthesis (e.g., using the Perkin Elmer ABI 431A Peptide Synthesizer in accordance with the instructions provided by the manufacturer). When full length protein is desired, shorter polypeptides may be fused by condensation of the amino terminus of one molecule with the carboxyl terminus of the other molecule to form a peptide bond.

The newly synthesized peptide can be substantially purified, for example, by preparative high performance liquid chromatography (e.g., Creighton,

PROTEINS, STRUCTURES AND MOLECULAR PRINCIPLES, WH Freeman and Co, New York NY [1983]). The composition of the synthetic peptides (or any other peptides or polypeptides of the invention) may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; Creighton, *supra*). Importantly, the amino acid sequence of hTRT, or any part thereof, may be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins or otherwise, or any part thereof or for any purpose, to produce a variant polypeptide of the invention.

2) RECOMBINANT EXPRESSION OF hTRT AND OTHER TRT PROTEINS

The present invention provides methods, reagents, vectors, and cells useful for expression of hTRT polypeptides and nucleic acids using *in vitro* (cell-free), *ex vivo* or *in vivo* (cell or organism-based) recombinant expression systems. In one embodiment, expression of the hTRT protein, or fragment thereof, comprises inserting the coding sequence into an appropriate expression vector (*i.e.*, a vector that contains the necessary elements for the transcription and translation of the inserted coding sequence required for the expression system employed). Thus, in one aspect, the invention provides for a polynucleotide substantially identical in sequence to an hTRT gene coding sequence at least 25 nucleotides, and preferably for many applications 50 to 100 nucleotides or more, of the hTRT cDNAs or genes of the invention, which is operably linked to a promoter to form a transcription unit capable of expressing an hTRT polypeptide. Methods well known to those skilled in the art can be used to construct the expression vectors containing an hTRT sequence and appropriate transcriptional or translational controls provided by the present invention (see, e.g., Sambrook et al., *supra*, Ausubel et al. *supra*, and this disclosure).

The hTRT polypeptides provided by the invention include fusion proteins that contain hTRT polypeptides or fragments of the hTRT protein. The fusion proteins are typically produced by recombinant means, although they may also be made by chemical synthesis. Fusion proteins can be useful in providing enhanced expression of the hTRT polypeptide constructs, or in producing hTRT polypeptides having other

desirable properties, for example, comprising a label (such as an enzymatic reporter group), binding group, or antibody epitope. An exemplary fusion protein, comprising hTRT and enhanced green fluorescent protein (EGFP) sequences is described in Example 15, *infra*. It will be apparent to one of skill that the uses and applications discussed in Example 15 and elsewhere herein are not limited to the particular fusion protein, but are illustrative of the uses of various fusion constructs.

The fusion protein systems of the invention can also be used to facilitate efficient production and isolation of hTRT proteins or peptides. For example, in some embodiments, the non-hTRT sequence portion of the fusion protein comprises a short peptide that can be specifically bound to an immobilized molecule such that the fusion protein can be separated from unbound components (such as unrelated proteins in a cell lysate). One example is a peptide sequence that is bound by a specific antibody. Another example is a peptide comprising polyhistidine tracts e.g. (His)₆ or histidine-tryptophan sequences that can be bound by a resin containing nickel or copper ions (i.e., metal-chelate affinity chromatography). Other examples include Protein A domains or fragments, which allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp. Seattle WA). In some embodiments, the fusion protein includes a cleavage site so that the hTRT or other TRT polypeptide sequence can be easily separated from the non-hTRT peptide or protein sequence. In this case, cleavage may be chemical (e.g., cyanogen bromide, 2-(2-nitrophenylsulphenyl)-3-methyl-3'-bromoindolene, hydroxylamine, or low pH) or enzymatic (e.g., Factor Xa, enterokinase). The choice of the fusion and cleavage systems may depend, in part, on the portion (i.e., sequence) of the hTRT polypeptide being expressed. Fusion proteins generally are described in Ausubel et al., *supra*, Ch. 16, Kroll et al., 1993, *DNA Cell. Biol.* 12:441, and the Invitrogen 1997 Catalog (Invitrogen Inc, San Diego CA). Other exemplary fusion proteins of the invention with epitope tags or tags and cleavage sites are provided in Example 6. *infra*.

It will be appreciated by those of skill that, although the expression systems discussed in this section are focused on expression of hTRT polypeptides, the same or similar cells, vectors and methods may be used to express hTRT

polynucleotides of the invention, including sense and antisense polynucleotides without necessarily desiring production of hTERT polypeptides. Typically, expression of a polypeptide requires a suitable initiation codon (e.g., methionine), open reading frame, and translational regulatory signals (e.g., a ribosome binding site, a termination codon) which may be omitted when translation of a nucleic acid sequence to produce a protein is not desired.

Expression of hTERT polypeptides and polynucleotides may be carried out to accomplish any of several related benefits provided by the present invention. One illustrative benefit is expression of hTERT polypeptides that are subsequently isolated from the cell in which they are expressed (for example for production of large amounts of hTERT for use as a vaccine or in screening applications to identify compounds that modulate telomerase activity). A second illustrative benefit is expression of hTERT in a cell to change the phenotype of the cell (as in gene therapy applications). Nonmammalian cells can be used for expression of hTERT for purification, while eukaryotic especially mammalian cells (e.g., human cells) can be used not only for isolation and purification of hTERT but also for expression of hTERT when a change in phenotype in a cell is desired (e.g., to effect a change in proliferative capacity as in gene therapy applications). By way of illustration and not limitation, hTERT polypeptides having one or more telomerase activities (e.g., telomerase catalytic activity) can be expressed in a host cell to increase the proliferative capacity of a cell (e.g., immortalize a cell) and, conversely, hTERT antisense polynucleotides or inhibitory polypeptides typically can be expressed to reduce the proliferative capacity of a cell (e.g., of a telomerase positive malignant tumor cell). Numerous specific applications are described herein, e.g., in the discussion of uses of the reagents and methods of the invention for therapeutic applications, below.

Illustrative useful expression systems (cells, regulatory elements, vectors and expression) of the present invention include a number of cell-free systems such as reticulocyte lysate and wheat germ systems using hTERT polynucleotides in accordance with general methods well known in the art (see, e.g., Ausubel et al. *supra* at Ch. 10). In alternative embodiments, the invention provides reagents and methods for expressing hTERT in prokaryotic or eukaryotic cells. Thus, the present invention provides nucleic

acids encoding hTRT polynucleotides, proteins, protein subsequences, or fusion proteins that can be expressed in bacteria, fungi, plant, insect, and animal, including human cell expression systems known in the art, including isolated cells, cell lines, cell cultures, tissues, and whole organisms. As will be understood by those of skill, the hTRT polynucleotides introduced into a host cell or cell free expression system will usually be operably linked to appropriate expression control sequences for each host or cell free system.

Useful bacterial expression systems include *E. coli*, bacilli (such as *Bacillus subtilis*), other enterobacteriaceae (such as *Salmonella*, *Serratia*, and various *Pseudomonas species*) or other bacterial hosts (e.g., *Streptococcus cremoris*, *Streptococcus lactis*, *Streptococcus thermophilus*, *Leuconostoc citrovorum*, *Leuconostoc mesenteroides*, *Lactobacillus acidophilus*, *Lactobacillus lactis*, *Bifidobacterium bifidum*, *Bifidobacteriu breve*, and *Bifidobacterium longum*). The hTRT expression constructs useful in prokaryotes include recombinant bacteriophage, plasmid or cosmid DNA expression vectors, or the like, and typically include promoter sequences. Illustrative promoters include inducible promoters, such as the *lac* promoter, the hybrid *lacZ* promoter of the Bluescript7 phagemid [Stratagene, La Jolla CA] or pSport1 [Gibco BRL]; phage lambda promoter systems; a tryptophan (*trp*) promoter system; and *ptp-lac* hybrids and the like. Bacterial expression constructs optionally include a ribosome binding site and transcription termination signal regulatory sequences. Illustrative examples of specific vectors useful for expression include, for example, pTrcHis2, (Invitrogen, San Diego CA), pThioHis A, B & C, and numerous others known in the art or that may be developed (see, e.g. Ausubel). Useful vectors for bacteria include those that facilitate production of hTRT- fusion proteins. Useful vectors for high level expression of fusion proteins in bacterial cells include, but are not limited to, the multifunctional *E. coli* cloning and expression vectors such as Bluescript7 (Stratagene), noted above, in which the sequence encoding hTRT protein, an hTRT fusion protein or an hTRT fragment may be ligated into the vector in-frame with sequences for the amino-terminal Met and the subsequent 7 residues of β -galactosidase so that a hybrid protein is produced (e.g., pIN vectors: Van Heeke and Schuster, 1989, *J. Biol. Chem.*, 264:5503). Vectors such as pGEX vectors (e.g., pGEX-

2TK; Pharmacia Biotech) may also be used to express foreign polypeptides, such as hTERT protein, as fusion proteins with glutathione S-transferase (GST). Such fusion proteins may be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems often include enterokinase, thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will, as may be useful in purification or other applications. Other examples are fusion proteins comprising hTERT and the *E. coli* Maltose Binding Protein (MBP) or *E. Coli* thioredoxin. Illustrative examples of hTERT expression constructs useful in bacterial cells are provided in Example 6, *infra*.

The invention further provides hTERT polypeptides expressed in fungal systems, such as *Dictyostelium* and, preferably, yeast, such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Torulopsis holmii*, *Saccharomyces fragilis*, *Saccharomyces lactis*, *Hansenula polymorpha* and *Candida pseudotropicalis*. When hTERT is expressed in yeast, a number of suitable vectors are available, including plasmid and yeast artificial chromosomes (YACs) vectors. The vectors typically include expression control sequences, such as constitutive or inducible promoters (e.g., such as alpha factor, alcohol oxidase, PGH, and 3-phosphoglycerate kinase or other glycolytic enzymes), and an origin of replication, termination sequences and the like, as desired. Suitable vectors for use in *Pichia* include pPICZ, His6/pPICZB, pPICZalpha, pPIC3.5K, pPIC9K, pA0815, pGAP2A, B & C, pGAP2alpha A, B, and C (Invitrogen, San Diego, CA) and numerous others known in the art or to be developed. In one embodiment, the vector His6/pPICZB (Invitrogen, San Diego, CA) is used to express a His₆-hTERT fusion protein in the yeast *Pichia pastoris*. An example of a vector useful in *Saccharomyces* is pYES2 (Invitrogen, San Diego, CA). Illustrative examples of hTERT expression constructs useful in yeast are provided in Example 6, *infra*.

The hTERT polypeptides of the invention may also be expressed in plant cell systems transfected with plant or plant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with bacterial expression vectors (e.g., Ti or pBR322 plasmid). In cases where plant virus expression vectors are used, the expression of an hTERT-encoding sequence may be driven by any

of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV (Brisson et al., 1984, *Nature* 310:511-514) may be used alone or in combination with the omega leader sequence from TMV (Takamatsu et al., 1987, *EMBO J.*, 6:307-311). Alternatively, plant promoters such as that from the small subunit gene of RUBISCO (Coruzzi et al., 1984, *EMBO J.*, 3:1671-1680; Broglie et al., 1984, *Science* 224:838-843) or heat shock promoters (Winter and Sinibaldi, 1991, *Results Probl. Cell Differ.*, 17:85), or storage protein gene promoters may be used. These constructs can be introduced into plant cells by direct DNA transformation or pathogen-mediated transfection (for reviews of such techniques, see Hobbs or Murry, 1992, in MCGRAW HILL YEARBOOK OF SCIENCE AND TECHNOLOGY McGraw Hill New York NY, pp. 191-196 [1992]; or Weissbach and Weissbach, 1988, METHODS FOR PLANT MOLECULAR BIOLOGY, Academic Press, New York NY, pp. 421-463).

Another expression system provided by the invention for expression of hTRT protein is an insect system. A preferred system uses a baculovirus polyhedrin promoter. In one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae. The sequence encoding the gene of interest may be cloned into a nonessential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of the sequence, e.g., encoding the hTRT protein, will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant viruses are then used to infect *S. frugiperda* cells or *Trichoplusia* larvae, in which the hTRT sequence is then expressed (see, for general methods, Smith et al., *J. Virol.*, 46:584 [1983]; Engelhard et al., *Proc. Natl. Acad. Sci.* 91:3224-7 [1994]). Useful vectors for baculovirus expression include pBlueBacHis2 A, B & C, pBlueBac4.5, pMelBacB and numerous others known in the art or to be developed. Illustrative examples of hTRT expression constructs useful in insect cells are provided in Example 6, *infra*.

The present invention also provides expression systems in mammals and mammalian cells. As noted *supra*, hTRT polynucleotides may be expressed in mammalian cells (e.g., human cells) for production of significant quantities of hTRT polypeptides (e.g., for purification) or to change the phenotype of a target cell (e.g., for

purposes of gene therapy, cell immortalization, or other). In the latter case, the hTERT polynucleotide expressed may or may not encode a polypeptide with a telomerase catalytic activity. That is, expression may be of a sense or antisense polynucleotide, an inhibitory or stimulatory polypeptide, a polypeptide with zero, one or more telomerase activities, and other combinations and variants disclosed herein or apparent to one of skill upon review of this disclosure.

Suitable mammalian host tissue culture cells for expressing the nucleic acids of the invention include any normal mortal or normal or abnormal immortal animal or human cell, including: monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293; Graham et al., *J. Gen. Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); CHO (ATCC CCL 61 and CRL 9618); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL 1587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather, et al., *Annals N.Y. Acad. Sci.* 383:44-46 (1982); MDCK cells (ATCC CCL 34 and CRL 6253); HEK 293 cells (ATCC CRL 1573); and W1-38 cells (ATCC CCL 75; ATCC: American Type Culture Collection, Rockville, MD). The use of mammalian tissue cell culture to express polypeptides is discussed generally in Winnacker, *FROM GENES TO CLONES* (VCH Publishers, N.Y., N.Y., 1987).

For mammalian host cells, viral-based and nonviral expression systems are provided. Nonviral vectors and systems include plasmids and episomal vectors, typically with an expression cassette for expressing a protein or RNA, and human artificial chromosomes (see, e.g., Harrington et al., 1997, *Nat Genet* 15:345). For example, nonviral vectors useful for expression of hTERT polynucleotides and polypeptides in mammalian (e.g., human) cells include pcDNA3.1/His, pEBVHis A, B & C, (Invitrogen, San Diego CA), MPSV vectors, others described in the Invitrogen 1997 Catalog (Invitrogen Inc, San Diego CA), which is incorporated in its entirety herein, and numerous others known in the art for other proteins. Illustrative examples

of hTERT expression constructs useful in mammalian cells are provided in Example 6, *infra*.

Useful viral vectors include vectors based on retroviruses, adenoviruses, adenoassociated viruses, herpes viruses, vectors based on SV40, papilloma virus, HBP
5 Epstein Barr virus, vaccinia virus vectors and Semliki Forest virus (SFV). SFV and vaccinia vectors are discussed generally in Ausubel et al., *supra*, Ch 16. These vectors are often made up of two components, a modified viral genome and a coat structure surrounding it (see generally Smith, 1995, *Annu. Rev. Microbiol.* 49: 807), although sometimes viral vectors are introduced in naked form or coated with proteins other than
10 viral proteins. However, the viral nucleic acid in a vector may be changed in many ways, for example, when designed for gene therapy. The goals of these changes are to disable growth of the virus in target cells while maintaining its ability to grow in vector form in available packaging or helper cells, to provide space within the viral genome for insertion of exogenous DNA sequences, and to incorporate new sequences that
15 encode and enable appropriate expression of the gene of interest. Thus, vector nucleic acids generally comprise two components: essential cis-acting viral sequences for replication and packaging in a helper line and the transcription unit for the exogenous gene. Other viral functions are expressed in trans in a specific packaging or helper cell line. Adenoviral vectors (e.g., for use in human gene therapy) are described in, e.g.,
20 Rosenfield et al., 1992, *Cell* 68: 143; PCT publications WO 94/12650; 94/12649; and 94/12629. In cases where an adenovirus is used as an expression vector, a sequence encoding hTERT may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a nonessential E1 or E3 region of the viral genome will result in a viable virus capable of
25 expressing in infected host cells (Logan and Shenk, 1984, *Proc. Natl. Acad. Sci.*, 81:3655). Replication-defective retroviral vectors harboring a therapeutic polynucleotide sequence as part of the retroviral genome are described in, e.g., Miller et al., 1990, *Mol. Cell. Biol.* 10: 4239; Kolberg, 1992, *J. NIH Res.* 4: 43; and Cometta et al., 1991, *Hum. Gene Ther.* 2: 215.

30 In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are often appropriate. Suitable promoters may be constitutive, cell

type-specific, stage-specific, and/or modulatable or regulatable (e.g., by hormones such as glucocorticoids). Useful promoters include, but are not limited to, the metallothionein promoter, the constitutive adenovirus major late promoter, the dexamethasone-inducible MMTV promoter, the SV40 promoter, the MRP polIII promoter, the constitutive MPSV promoter, the tetracycline-inducible CMV promoter (such as the human immediate-early CMV promoter), the constitutive CMV promoter, and promoter-enhancer combinations known in the art.

Other regulatory elements may also be required or desired for efficient expression of an hTERT polynucleotide and/or translation of a sequence encoding hTERT proteins. For translation, these elements typically include an ATG initiation codon and adjacent ribosome binding site or other sequences. For sequences encoding the hTERT protein, provided its initiation codon and upstream promoter sequences are inserted into an expression vector, no additional translational or other control signals may be needed. However, in cases where only coding sequence, or a portion thereof, is inserted, exogenous transcriptional and/or translational control signals (e.g., the promoter, ribosome-binding site, and ATG initiation codon) must often be provided. Furthermore, the initiation codon must typically be in the correct reading frame to ensure translation of the desired protein. Exogenous transcriptional elements and initiation codons can be of various origins, both natural and synthetic. In addition, the efficiency of expression may be enhanced by the inclusion of enhancers appropriate to the cell system in use (Scharf et al., 1994, *Results Probl. Cell Differ.* 20:125; and Bittner et al. 1987, *Meth. Enzymol.*, 153:516). For example, the SV40 enhancer or CMV enhancer may be used to increase expression in mammalian host cells.

Expression of hTERT gene products can also be effected (increased) by activation of an hTERT promoter or enhancer in a cell such as a human cell, e.g., a telomerase-negative cell line. Activation can be carried out in a variety of ways, including administration of an exogenous promoter activating agent, or inhibition of a cellular component that suppresses expression of the hTERT gene. It will be appreciated that, conversely, inhibition of promoter function, as described *infra*, will reduce hTERT gene expression.

The invention provides inducible and repressible expression of hTERT

polypeptides using such system as the Ecdysone-Inducible Expression System (Invitrogen), and the Tet-On and Tet-off tetracycline regulated systems from Clontech. The ecdysone-inducible expression system uses the steroid hormone ecdysone analog, muristerone A, to activate expression of a recombinant protein via a heterodimeric nuclear receptor (No et al., 1996, *Proc. Natl. Acad. Sci. USA* 93:3346). In one embodiment of the invention, hTERT is cloned in the pIND vector (Clontech), which contains five modified ecdysone response elements (E/GREs) upstream of a minimal heat shock promoter and the multiple cloning site. The construct is then transfected in cell lines stably expressing the ecdysone receptor. After transfection, cells are treated with muristerone A to induce intracellular expression from pIND. In another embodiment of the invention, hTERT polypeptide is expressed using the Tet-on and Tet-off expression systems (Clontech) to provide regulated, high-level gene expression (Gossen et al., 1992, *Proc. Natl. Acad. Sci. USA* 89:5547; Gossen et al., 1995, *Science* 268:1766).

The hTERT vectors of the invention may be introduced into a cell, tissue, organ, patient or animal by a variety of methods. The nucleic acid expression vectors (typically dsDNA) of the invention can be transferred into the chosen host cell by well-known methods such as calcium chloride transformation (for bacterial systems), electroporation, calcium phosphate treatment, liposome-mediated transformation, injection and microinjection, ballistic methods, virosomes, immunoliposomes, polycation:nucleic acid conjugates, naked DNA, artificial virions, fusion to the herpes virus structural protein VP22 (Elliot and O'Hare, *Cell* 88:223), agent-enhanced uptake of DNA, and *ex vivo* transduction. Useful liposome-mediated DNA transfer methods are described in US Patent Nos. 5,049,386, US 4,946,787, and US 4,897,355; PCT publications WO 91/17424, WO 91/16024; Wang and Huang, 1987, *Biochem. Biophys. Res. Commun.* 147: 980; Wang and Huang, 1989, *Biochemistry* 28: 9508; Litzinger and Huang, 1992, *Biochem. Biophys. Acta* 1113:201; Gao and Huang, 1991, *Biochem. Biophys. Res. Commun.* 179: 280. Immunoliposomes have been described as carriers of exogenous polynucleotides (Wang and Huang, 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:7851; Trubetskoy et al., 1992, *Biochem. Biophys. Acta* 1131:311) and may have improved cell type specificity as compared to liposomes by virtue of the inclusion of

specific antibodies which presumably bind to surface antigens on specific cell types.

Behr et al., 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86:6982 report using lipopolyamine as a reagent to mediate transfection itself, without the necessity of any additional phospholipid to form liposomes. Suitable delivery methods will be selected by practitioners in view of acceptable practices and regulatory requirements (e.g., for gene therapy or production of cell lines for expression of recombinant proteins). It will be appreciated that the delivery methods listed above may be used for transfer of nucleic acids into cells for purposes of gene therapy, transfer into tissue culture cells, and the like.

For long-term, high-yield production of recombinant proteins, stable expression will often be desired. For example, cell lines which stably express hTRT can be prepared using expression vectors of the invention which contain viral origins of replication or endogenous expression elements and a selectable marker gene.

Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth of cells which successfully express the introduced sequences in selective media.

Resistant, stably transfected cells can be proliferated using tissue culture techniques appropriate to the cell type. An amplification step, e.g., by administration of methyltrexate to cells transfected with a DHFR gene according to methods well known in the art, can be included.

In addition, a host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, phosphorylation, lipidation and acylation. Post-translational processing may also be important for correct insertion, folding and/or function.

Different host cells have cellular machinery and characteristic mechanisms specific for each cell for such post-translational activities and so a particular cell may be chosen to ensure the correct modification and processing of the introduced, foreign protein.

The present invention also provides transgenic animals (i.e., mammals transgenic for a human or other TRT gene sequence) expressing an hTRT or other TRT

polynucleotide or polypeptide. In one embodiment, hTRT is secreted into the milk of a transgenic mammal such as a transgenic bovine, goat, or rabbit. Methods for production of such animals are found, e.g., in Heyneker et al., PCT WO 91/08216.

5 The hTRT proteins and complexes of the invention, including those made using the expression systems disclosed herein *supra*, may be purified using a variety of general methods known in the art in accordance with the specific methods provided by the present invention (e.g., *infra*). One of skill in the art will recognize that after chemical synthesis, biological expression, or purification, the hTRT protein may possess a conformation different than a native conformation of naturally occurring
10 telomerase. In some instances, it may be helpful or even necessary to denature (e.g., including reduction of disulfide or other linkages) the polypeptide and then to cause the polypeptide to re-fold into the preferred conformation. Productive refolding may also require the presence of hTR (or hTR fragments). Methods of reducing and denaturing proteins and inducing re-folding are well known to those of skill in the art (see, e.g.,
15 Debinski et al., 1993, *J. Biol. Chem.*, 268:14065; Kreitman and Pastan, 1993, *Bioconjug. Chem.*, 4:581; and Buchner et al., 1992, *Anal. Biochem.*, 205:263; and McCaman et al., 1985, *J. Biotech.* 2:177). See also PCT Publication WO 96 40868, *supra*.

20 **D) COMPLEXES OF HUMAN TRT AND HUMAN TELOMERASE RNA, TELOMERASE-ASSOCIATED PROTEINS, AND OTHER BIOMOLECULES PRODUCED BY COEXPRESSION AND OTHER MEANS**

hTRT polypeptides of the invention can associate *in vivo* and *in vitro* with other biomolecules, including RNAs (e.g., hTR), proteins (e.g., telomerase-associated proteins), DNA (e.g., telomeric DNA, $[T_2AG_3]_N$), and nucleotides, such as
25 (deoxy)ribonucleotide triphosphates. These associations can be exploited to assay hTRT presence or function, to identify or purify hTRT or telomerase-associated molecules, and to analyze hTRT or telomerase structure or function in accordance with the methods of the present invention.

30 In one embodiment, the present invention provides hTRT complexed with (e.g., associated with or bound to) a nucleic acid, usually an RNA, for example to

produce a telomerase holoenzyme. In one embodiment, the bound RNA is capable of acting as a template for telomerase-mediated DNA synthesis. Examples of RNAs that may be complexed with the hTERT polypeptide include a naturally occurring host cell telomerase RNA, a human telomerase RNA (e.g., hTR; U.S. Patent No. 5,583,016), an hTR subsequence or domain, a synthetic RNA, or other RNAs. The RNA-hTERT protein complex (an RNP) typically exhibits one or more telomerase activities, such as telomerase catalytic activities. These hTERT-hTR RNPs (or other hTERT-RNA complexes) can be produced by a variety of methods, as described *infra* for illustrative purposes, including *in vitro* reconstitution, by co-expression of hTERT and hTR (or other RNA) *in vitro* (i.e., in a cell free system), *in vivo* reconstitution, or *ex vivo* reconstitution.

Thus, the present invention provides, in one embodiment, an hTERT-hTR complex (or other hTERT-RNA complex) formed *in vitro* by mixing separately purified components ("*in vitro* reconstitution;" see, e.g., U.S. Patent No. 5,583,016 for a description of reconstitution; also see Autexier et al., *EMBO J.* 15:5928).

In an alternative embodiment, the invention provides telomerase RNPs produced by coexpression of the hTERT polypeptide and an RNA (e.g., hTR) *in vitro* in a cell-free transcription-translation system (e.g. wheat germ or rabbit reticulocyte lysate). As shown in Example 7, *in vitro* co-expression of a recombinant hTERT polypeptide and hTR results in production of telomerase catalytic activity (as measured by a TRAP assay).

Further provided by the present invention are telomerase RNPs produced by expression of the hTERT polypeptide in a cell, e.g., a mammalian cell, in which hTR is naturally expressed or in which hTR (or another RNA capable of forming a complex with the hTERT protein) is introduced or expressed by recombinant means. Thus, in one embodiment, hTERT is expressed in a telomerase negative human cell in which hTR is present (e.g., BJ or IMP90 cells), allowing the two molecules to assemble into an RNP. In another embodiment, hTERT is expressed in a human or non-human cell in which hTR is recombinantly expressed. Methods for expression of hTR in a cell are found in U.S. Patent 5,583,016. Further, a clone containing a cDNA encoding the RNA component of telomerase has been placed on deposit as pGRN33 (ATCC 75926).

Genomic sequences encoding the RNA component of human telomerase are also on deposit in the ~15 kb *Sau*III A1 to *Hind*III insert of lambda clone 28-1 (ATCC 75925). For expression in eukaryotic cells the hTERT sequence will typically be operably linked to a transcription initiation sequence (RNA polymerase binding site) and transcription terminator sequences (see, e.g., PCT Publication WO 96/01835; Feng et al., 1995, *Science* 269:1236).

The present invention further provides recombinantly produced or substantially purified hTERT polypeptides coexpressed and/or associated with so-called "telomerase-associated proteins." Thus, the present invention provides hTERT coexpressed with, or complexed with, other proteins (e.g., telomerase-associated proteins). Telomerase-associated proteins are those proteins that copurify with human telomerase and/or that may play a role in modulating telomerase function or activity, for example by participating in the association of telomerase with telomeric DNA. Examples of telomerase-associated proteins include (but are not limited to) the following proteins and/or their human homologs: nucleolin (see, Srivastava et al., 1989, *FEBS Letts.* 250:99); EF2H (elongation factor 2 homolog; see Nomura et al. 1994, *DNA Res. (Japan)* 1:27, GENBANK accession #D21163); TP1/TLP1 (Harrington et al., 1997, *Science* 275:973; Nakayama, 1997, *Cell* 88:875); the human homologue of the *Tetrahymena* p95 or p95 itself (Collins et al., 1995, *Cell* 81:677); TPC2 (a telomere length regulatory protein; ATCC accession number 97708; TPC3 (also a telomere length regulatory protein; ATCC accession number 97707; DNA-binding protein B (dbpB; Horwitz et al., 1994, *J. Biol. Chem.* 269:14130; and Telomere Repeat Binding Factors (TRF 1 & 2; Chang et al., 1995, *Science* 270:1663; Chong et al., 1997, *Hum Mol Genet* 6:69); EST1, 3 and 4 (Lendvay et al., 1996, *Genetics* 144:1399, Nugent et al., 1996, *Science* 274:249, Lundblad et al., 1989, *Cell* 57:633); and End-capping factor (Cardenas et al., 1993, *Genes Dev.* 7:883).

Telomerase associated proteins can be identified on the basis of co-purification with, or binding to, hTERT protein or the hTERT-hTR RNP. Alternatively, they can be identified on the basis of binding to an hTERT fusion protein, e.g., a GST-hTERT fusion protein or the like, as determined by affinity purification (see, Ausubel et al. Ch 20). A particularly useful technique for assessing protein-protein interactions.

which is applicable to identifying hTERT-associated proteins, is the two hybrid screen method of Chien et al. (*Proc. Natl. Acad. Sci. USA* 88:9578 [1991]; see also Ausubel et al., *supra*, at Ch. 20). This screen identifies protein-protein interactions *in vivo* through reconstitution of a transcriptional activator, the yeast Gal4 transcription protein (see, 5 Fields and Song, 1989, *Nature* 340:245. The method is based on the properties of the yeast Gal4 protein, which consists of separable domains responsible for DNA-binding and transcriptional activation. Polynucleotides, usually expression vectors, encoding two hybrid proteins are constructed. One polynucleotide comprises the yeast Gal4 DNA-binding domain fused to a polypeptide sequence of a protein to be tested for an 10 hTERT interaction (e.g., nucleolin or EF2H). Alternatively the yeast Gal4 DNA-binding domain is fused to cDNAs from a human cell, thus creating a library of human proteins fused to the Gal4 DNA binding domain for screening for telomerase associated proteins. The other polynucleotide comprises the Gal4 activation domain fused to an hTERT polypeptide sequence. The constructs are introduced into a yeast host cell. 15 Upon expression, intermolecular binding between hTERT and the test protein can reconstitute the Gal4 DNA-binding domain with the Gal4 activation domain. This leads to the transcriptional activation of a reporter gene (e.g., lacZ, HIS3) operably linked to a Gal4 binding site. By selecting for, or by assaying the reporter, gene colonies of cells that contain an hTERT interacting protein or telomerase associated 20 protein can be identified. Those of skill will appreciate that there are numerous variations of the 2-hybrid screen, e.g., the LexA system (Bartel et al. 1993, *in Cellular Interactions in Development: A Practical Approach* Ed. Hartley, D.A. (Oxford Univ. Press) pp. 153-79).

Another useful method for identifying telomerase-associated proteins is 25 a three-hybrid system (see, e.g., Zhang et al., 1996, *Anal. Biochem.* 242:68; Licitra et al., 1996, *Proc. Natl. Acad. Sci. USA* 93:12817). The telomerase RNA component can be utilized in this system with the TERT or hTERT protein and a test protein. Another useful method for identifying interacting proteins, particularly (i.e., proteins that heterodimerize or form higher order heteromultimers), is the *E. coli*/BCCP interactive 30 screening system (see, Germino et al. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:933; Guarente (1993) *Proc. Natl. Acad. Sci. (U.S.A.)* 90:1639).

The present invention also provides complexes of telomere binding proteins (which may or may not be telomerase associated proteins) and hTRT (which may or may not be complexed with hTR, other RNAs, or one or more telomerase associated proteins). Examples of telomere binding proteins include TRF1 and TRF2 (5 *supra*); mnpA1, mnpA2, RAP1 (Buchman et al., 1988, *Mol. Cell. Biol.* 8:210, Buchman et al., 1988, *Mol. Cell. Biol.* 8:5086), SIR3 and SIR4 (Aparicio et al., 1991, *Cell* 66:1279), TEL1 (Greenwell et al., 1995, *Cell* 82:823; Morrow et al., 1995, *Cell* 82:831); ATM (Savitsky et al., 1995, *Science* 268:1749), end-capping factor (Cardenas et al., 1993, *Genes Dev.* 7:883), and corresponding human homologs. The
10 aforementioned complexes may be produced generally as described *supra* for complexes of hTRT and hTR or telomerase associated proteins, e.g., by mixing or co-expression *in vitro* or *in vivo*.

V. ANTIBODIES AND OTHER BINDING AGENTS

15 In a related aspect, the present invention provides antibodies that are specifically immunoreactive with hTRT, including polyclonal and monoclonal antibodies, antibody fragments, single chain antibodies, human and chimere antibodies, including antibodies or antibody fragments fused to phage coat or cell surface proteins, and others known in the art and described herein. The antibodies of
20 the invention can specifically recognize and bind polypeptides that have an amino acid sequence that is substantially identical to the amino acid sequence set forth in Figure 17 (SEQUENCE ID NO: 2), or an immunogenic fragment thereof or epitope on the protein defined thereby. The antibodies of the invention can exhibit a specific binding affinity for hTRT of at least about 10^7 , 10^8 , 10^9 , or 10^{10} M⁻¹, and may be polyclonal,
25 monoclonal, recombinant or otherwise produced. The invention also provides anti-hTRT antibodies that recognize an hTRT conformational epitope (e.g., an epitope on the surface of the hTRT protein or a telomerase RNP). Likely conformational epitopes can be identified, if desired, by computer-assisted analysis of the hTRT protein sequence, comparison to the conformation of related reverse transcriptases, such as the
30 p66 subunit of HIV-1 (see, e.g., Figure 3), or empirically. Anti-hTRT antibodies that recognize conformational epitopes have utility, *inter alia*, in detection and purification

of human telomerase and in the diagnosis and treatment of human disease.

For the production of anti-hTERT antibodies, hosts such as goats, sheep, cows, guinea pigs, rabbits, rats, or mice, may be immunized by injection with hTERT protein or any portion, fragment or oligopeptide thereof which retains immunogenic properties. In selecting hTERT polypeptides for antibody induction, one need not retain biological activity; however, the protein fragment, or oligopeptide must be immunogenic, and preferably antigenic. Immunogenicity can be determined by injecting a polypeptide and adjuvant into an animal (e.g., a rabbit) and assaying for the appearance of antibodies directed against the injected polypeptide (see, e.g., Harlow and Lane, ANTIBODIES: A LABORATORY MANUAL, COLD SPRING HARBOR LABORATORY, New York (1988), which is incorporated in its entirety and for all purposes, e.g., at Chapter 5). Peptides used to induce specific antibodies typically have an amino acid sequence consisting of at least five amino acids, preferably at least 8 amino acids, more preferably at least 10 amino acids. Usually they will mimic or have substantial sequence identity to all or a contiguous portion of the amino acid sequence of the protein of SEQUENCE ID NO: 2. Short stretches of hTERT protein amino acids may be fused with those of another protein, such as keyhole limpet hemocyanin, and an anti-hTERT antibody produced against the chimeric molecule. Depending on the host species, various adjuvants may be used to increase immunological response.

The antigen is presented to the immune system in a fashion determined by methods appropriate for the animal. These and other parameters are generally well known to immunologists. Typically, injections are given in the footpads, intramuscularly, intradermally, perilymph nodally or intraperitoneally. The immunoglobulins produced by the host can be precipitated, isolated and purified by routine methods, including affinity purification.

Illustrative examples of immunogenic hTERT peptides include are provided in Example 8. In addition, Example 8 describes the production and use of anti-hTERT polyclonal antibodies.

30 A) MONOCLONAL ANTIBODIES

Monoclonal antibodies to hTERT proteins and peptides may be prepared

in accordance with the methods of the invention using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Koehler and Milstein (*Nature* 256:495 [1975]), the human B-cell hybridoma technique (Kosbor et al., 1983, *Immunol. Today* 4:72; Cote et al., 1983, *Proc. Natl. Acad. Sci. USA*, 80:2026), and the EBV-hybridoma technique (Cole et al., *MONOCLONAL ANTIBODIES AND CANCER THERAPY*, Alan R Liss Inc, New York NY, pp 77-96 [1985]).

In one embodiment, appropriate animals are selected and the appropriate immunization protocol followed. The production of non-human monoclonal antibodies, e.g., murine, lagomorpha, equine, is well known and can be accomplished by, for example, immunizing an animal with a preparation containing hTERT or fragments thereof. In one method, after the appropriate period of time, the spleens of the animals are excised and individual spleen cells are fused, typically, to immortalized myeloma cells under appropriate selection conditions. Thereafter, the cells are clonally separated and the supernatants of each clone (e.g., hybridoma) are tested for the production of an appropriate antibody specific for the desired region of the antigen. Techniques for producing antibodies are well known in the art. See, e.g., Goding et al., *MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE* (2D ED.) Acad. Press, N.Y., and Harlow and Lane, *supra*, each of which is incorporated in its entirety and for all purposes. Other suitable techniques involve the *in vitro* exposure of lymphocytes to the antigenic polypeptides or alternatively, to selection of libraries of antibodies in phage or similar vectors (see, *infra*).

B) HUMAN ANTIBODIES

In another aspect of the invention, human antibodies against an hTERT polypeptide are provided. Human monoclonal antibodies against a known antigen can also be made using transgenic animals having elements of a human immune system (see, e.g., U.S. Patent Nos. 5,569,825 and 5,545,806, both of which are incorporated by reference in their entirety for all purposes) or using human peripheral blood cells (Casali et al., 1986, *Science* 234:476). Some human antibodies are selected by competitive binding experiments, or otherwise, to have the same epitope specificity as a

particular mouse antibody.

In an alternative embodiment, human antibodies to an hTERT polypeptide can be produced by screening a DNA library from human B cells according to the general protocol outlined by Huse et al., 1989, *Science* 246:1275, which is incorporated
5 by reference. Antibodies binding to the hTERT polypeptide are selected. Sequences encoding such antibodies (or binding fragments) are then cloned and amplified. The protocol described by Huse is often used with phage-display technology.

C) HUMANIZED OR CHIMERIC ANTIBODIES

10 The invention also provides anti-hTERT antibodies that are made chimeric, human-like or humanized, to reduce their potential antigenicity, without reducing their affinity for their target. Preparation of chimeric, human-like and humanized antibodies have been described in the art (see, e.g., U.S. Patent Nos. 5,585,089 and 5,530,101; Queen, et al., 1989, *Proc. Nat'l Acad. Sci. USA* 86:10029;
15 and Verhoeyan et al., 1988, *Science* 239:1534; each of which is incorporated by reference in their entirety and for all purposes). Humanized immunoglobulins have variable framework regions substantially from a human immunoglobulin (termed an acceptor immunoglobulin) and complementarity determining regions substantially from a non-human (e.g., mouse) immunoglobulin (referred to as the donor immunoglobulin).
20 The constant region(s), if present, are also substantially from a human immunoglobulin.

In some applications, such as administration to human patients, the humanized (as well as human) anti-hTERT antibodies of the present invention offer several advantages over antibodies from murine or other species: (1) the human immune system should not recognize the framework or constant region of the
25 humanized antibody as foreign, and therefore the antibody response against such an injected antibody should be less than against a totally foreign mouse antibody or a partially foreign chimeric antibody; (2) because the effector portion of the humanized antibody is human, it may interact better with other parts of the human immune system; and (3) injected humanized antibodies have a half-life essentially equivalent to
30 naturally occurring human antibodies, allowing smaller and less frequent doses than antibodies of other species. As implicit from the foregoing, anti hTERT antibodies have

application in the treatment of disease, i.e., to target telomerase-positive cells.

D) PHAGE DISPLAY

The present invention also provides anti-hTERT antibodies (or binding
5 compositions) produced by phage display methods (see, e.g., Dower et al., WO
91/17271 and McCafferty et al., WO 92/01047; and Vaughan *et al.*, 1996, *Nature
Biotechnology*, 14: 309; each of which is incorporated by reference in its entirety for all
purposes). In these methods, libraries of phage are produced in which members display
different antibodies on their outer surfaces. Antibodies are usually displayed as Fv or
10 Fab fragments. Phage displaying antibodies with a desired specificity are selected by
affinity enrichment to an hTERT polypeptide.

In a variation of the phage-display method, humanized antibodies
having the binding specificity of a selected murine antibody can be produced. In this
method, either the heavy or light chain variable region of the selected murine antibody
15 is used as a starting material. If, for example, a light chain variable region is selected as
the starting material, a phage library is constructed in which members display the same
light chain variable region (i.e., the murine starting material) and a different heavy
chain variable region. The heavy chain variable regions are obtained from a library of
rearranged human heavy chain variable regions. A phage showing strong specific
20 binding for the hTERT polypeptide (e.g., at least 10^4 and preferably at least 10^6 M⁻¹) is
selected. The human heavy chain variable region from this phage then serves as a
starting material for constructing a further phage library. In this library, each phage
displays the same heavy chain variable region (i.e., the region identified from the first
display library) and a different light chain variable region. The light chain variable
25 regions are obtained from a library of rearranged human variable light chain regions.
Again, phage showing strong specific binding are selected. These phage display the
variable regions of completely human anti-hTERT antibodies. These antibodies usually
have the same or similar epitope specificity as the murine starting material.

E) HYBRID ANTIBODIES

The invention also provides hybrid antibodies that share the specificity

of antibodies against an hTERT polypeptide but are also capable of specific binding to a second moiety. In such hybrid antibodies, one heavy and light chain pair is usually from an anti-hTERT antibody and the other pair from an antibody raised against another epitope or protein. This results in the property of multi-functional valency, i.e., ability to bind at least two different epitopes simultaneously, where at least one epitope is the epitope to which the anti-complex antibody binds. Such hybrids can be formed by fusion of hybridomas producing the respective component antibodies, or by recombinant techniques. Such hybrids can be used to carry a compound (i.e., drug) to a telomerase-positive cell (i.e., a cytotoxic agent is delivered to a cancer cell).

Immunoglobulins of the present invention can also be fused to functional regions from other genes (e.g., enzymes) to produce fusion proteins (e.g., immunotoxins) having useful properties.

F) ANTI-IDIOTYPIC ANTIBODIES

Also useful are anti-idiotypic antibodies which can be isolated by the above procedures. Anti-idiotypic antibodies may be prepared by, for example, immunization of an animal with the primary antibody (i.e., anti-hTERT antibodies or hTERT-binding fragments thereof). For anti-hTERT antibodies, anti-idiotypic antibodies whose binding to the primary antibody is inhibited by an hTERT polypeptide or fragments thereof are selected. Because both the anti-idiotypic antibody and the hTERT polypeptide or fragments thereof bind the primary immunoglobulin, the anti-idiotypic immunoglobulin can represent the "internal image" of an epitope and thus can substitute for the hTERT polypeptide in assays or can be used to bind (i.e., inactivate) anti-hTERT antibodies, e.g., in a patient. Anti-idiotypic antibodies can also interact with telomerase associated proteins. Administration of such antibodies can affect telomerase function by titrating out or competing with hTERT in binding to hTERT-associated proteins.

G) GENERAL

The antibodies of the invention may be of any isotype, e.g., IgM, IgD, IgG, IgA, and IgE, with IgG, IgA and IgM often preferred. Humanized antibodies may comprise sequences from more than one class or isotype.

In another embodiment of the invention, fragments of the intact antibodies described above are provided. Typically, these fragments can compete with the intact antibody from which they were derived for specific binding to the hTERT polypeptide, and bind with an affinity of at least 10^7 , 10^8 , 10^9 M^{-1} , or 10^{10} M^{-1} .

5 Antibody fragments include separate heavy chains, light chains, Fab, Fab' F(ab')₂, Fabc, and Fv. Fragments can be produced by enzymatic or chemical separation of intact immunoglobulins. For example, a F(ab')₂ fragment can be obtained from an IgG molecule by proteolytic digestion with pepsin at pH 3.0-3.5 using standard methods such as those described in Harlow and Lane, *supra*. Fab fragments may be obtained
10 from F(ab')₂ fragments by limited reduction, or from whole antibody by digestion with papain in the presence of reducing agents (*see generally*, Paul, W., ed FUNDAMENTAL IMMUNOLOGY 2ND Raven Press, N.Y., 1989, Ch. 7, incorporated by reference in its entirety for all purposes). Fragments can also be produced by recombinant DNA techniques. Segments of nucleic acids encoding selected fragments are produced by
15 digestion of full-length coding sequences with restriction enzymes, or by *de novo* synthesis. Often fragments are expressed in the form of phage-coat fusion proteins.

Many of the immunoglobulins described above can undergo non-critical amino-acid substitutions, additions or deletions in both the variable and constant regions without loss of binding specificity or effector functions, or intolerable reduction
20 of binding affinity (i.e., below about 10^7 M^{-1}). Usually, immunoglobulins incorporating such alterations exhibit substantial sequence identity to a reference immunoglobulin from which they were derived. A mutated immunoglobulin can be selected having the same specificity and increased affinity compared with a reference immunoglobulin from which it was derived. Phage-display technology offers useful techniques for
25 selecting such immunoglobulins. *See*, e.g., Dower et al., WO 91/17271 McCafferty et al., WO 92/01047; and Huse, WO 92/06204.

The antibodies of the present invention can be used with or without modification. Frequently, the antibodies will be labeled by joining, either covalently or non-covalently, a detectable label. As labeled binding entities, the antibodies of the
30 invention are particularly useful in diagnostic applications.

The anti-hTERT antibodies of the invention can be purified using well

known methods. The whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention can be purified using the methods and reagents of the present invention in accordance with standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column
5 chromatography, gel electrophoresis and the like (*see generally* Scopes, PROTEIN PURIFICATION: PRINCIPLES AND PRACTICE 3RD EDITION (Springer-Verlag, N.Y., 1993)). Substantially pure immunoglobulins of at least about 90 to 95%, or even 98 to 99% or more homogeneity are preferred.

10 VI. PURIFICATION OF HUMAN TELOMERASE

The present invention provides isolated human telomerase of unprecedented purity. In particular, the present invention provides: purified hTERT of recombinant or nonrecombinant origin; purified hTERT-hTR complexes (i.e., RNPs) of recombinant, nonrecombinant, or mixed origin, optionally comprising one or more
15 telomerase-associated proteins; purified naturally occurring human telomerase; and the like. Moreover, the invention provides methods and reagents for partially, substantially or highly purifying the above-molecules and complexes, including variants, fusion proteins, naturally occurring proteins, and the like (collectively referred to as "hTERT and/or hTERT complexes").

20 Prior to the present disclosure, attempts had been made to purify the telomerase enzyme complex to homogeneity had met with limited success. The methods provided in the aforelisted applications provide purification of telomerase by approximately up to 60,000-fold or more compared to crude cell extracts. The present invention provides hTERT and hTERT complexes of even greater purity, in part by virtue
25 of the novel immunoaffinity reagents (e.g., anti-hTERT antibodies) of the present invention, and/or the reagents, cells, and methods provided herein for recombinant expression of hTERT. Recombinant expression of hTERT and hTERT complexes facilitates purification because the desired molecules can be produced at much higher levels than found in most expressing cells occurring in nature, and/or because the
30 recombinant hTERT molecule can be modified (e.g., by fusion with an epitope tag) such that it may be easily purified.

It will be recognized that naturally occurring telomerase can be purified from any telomerase-positive cell, and recombinant hTERT and hTERT complexes can be expressed and purified, *inter alia*, using any of the *in vitro*, *in vivo*, *ex vivo*, or plant or animal expression systems disclosed *supra*, or others/systems known in the art.

5 In one embodiment, the hTERT, telomerase and other compositions of the invention are purified using an immunoaffinity step, alone or in combination with other purification steps. Typically, an immobilized or immobilizable anti-hTERT antibody, as provided by the present invention, is contacted with a sample, such as a cell lysate, that contains the desired hTERT or hTERT-containing complex under conditions in which
10 anti-hTERT antibody binds the hTERT antigen. After removal of the unbound components of the sample by methods well known in the art, the hTERT composition may be eluted, if desired, from the antibody, in substantially pure form. In one embodiment, immunoaffinity chromatography methods well known in the art are used (see, e.g., Harlow and Lane, *supra*; and Ausubel, *supra*; Hermanson et al., 1992,
15 IMMOBILIZED AFFINITY LIGAND TECHNIQUES (Academic Press, San Diego)) in accordance with the methods of the invention. In another illustrative embodiment, immunoprecipitation of anti-hTERT-immunoglobulin-hTERT complexes is carried out using immobilized Protein A. Numerous variations and alternative immunoaffinity purification protocols suitable for use in accordance with the methods and reagents of
20 the invention are well-known to those of skill.

In another embodiment, recombinant hTERT proteins can, as a consequence of their high level of expression, be purified using routine protein purification methods, such as ammonium sulfate precipitation, affinity columns (e.g., immunoaffinity), size-exclusion, anion and cation exchange chromatography, gel
25 electrophoresis and the like (see, generally, R. Scopes, PROTEIN PURIFICATION, Springer-Verlag, N.Y. (1982) and Deutscher, METHODS IN ENZYMOLOGY VOL. 182: GUIDE TO PROTEIN PURIFICATION, Academic Press, Inc. N.Y. (1990)) instead of, or in addition to, immunoaffinity methods. Cation exchange methods can be particularly useful due to the basic pI of the hTERT protein. For example, immobilized phosphate
30 may be used as a cation exchange functional group (e.g., P-11 Phosphocellulose, Whatman catalog #4071 or Cellulose Phosphate. Sigma catalog #C 3145).

Immobilized phosphate has two advantageous features for hTERT purification - it is a cation exchange resin, and it shows physical resemblance to the phosphate backbone of nucleic acid. This can allow for affinity chromatography because hTERT binds hTR and telomeric DNA. Other non-specific and specific nucleic acid affinity chromatography methods are also useful for purification (e.g., Alberts et al., 1971, *Methods Enzymol.* 21:198; Arnt-Jovin et al., 1975, *Eur. J. Biochem.* 54:411; Pharmacia catalog #27-5575-02). Further exploitation of this binding function of hTERT could include the use of specific nucleic acid (e.g., telomerase primer or hTR) affinity chromatography for purification (Chodosh et al., 1986, *Mol. Cell. Biol.* 6:4723; Wu et al., 1987, *Science* 238:1247; Kadonaga, 1991, *Methods Enzymol.* 208:10); immobilized Cibricon Blue Dye, which shows physical resemblance to nucleotides, is another useful resin for hTERT purification (Pharmacia catalog #17-0948-01 or Sigma catalog #C 1285), due to hTERT binding of nucleotides (e.g., as substrates for DNA synthesis).

In one embodiment, hTERT proteins are isolated directly from an *in vitro* or *in vivo* expression system in which other telomerase components are not coexpressed. It will be recognized that isolated hTERT protein may also be readily obtained from purified human telomerase or hTERT complexes, for example, by disrupting the telomerase RNP (e.g., by exposure to a mild or other denaturant) and separating the RNP components (e.g., by routine means such as chromatography or immunoaffinity chromatography).

Telomerase purification may be monitored using a telomerase activity assay (e.g., the TRAP assay, conventional assay, or primer-binding assay), by measuring the enrichment of hTERT (e.g., by ELISA), by measuring the enrichment of hTR, or other methods known in the art.

The purified human telomerase, hTERT proteins, and hTERT complexes provided by the present invention are, in one embodiment, highly purified (i.e., at least about 90% homogeneous, more often at least about 95% homogeneous). Homogeneity can be determined by standard means such as SDS-polyacrylamide gel electrophoresis and other means known in the art (see, e.g., Ausubel et al, *supra*). It will be understood that, although highly purified human telomerase, hTERT protein, or hTERT complexes are sometimes described, substantially purified (e.g., at least about 75% homogeneous) or

partially purified (e.g., at least about 20% homogeneous) human telomerase, hTERT protein, or hTERT complexes are useful in many applications, and are also provided by the present invention. For example, partially purified telomerase is useful for screening test compounds for telomerase modulatory activity, and other uses (see, *infra* and
5 *supra*; see U.S. Patent No. 5,645,986).

VII. TREATMENT OF TELOMERASE-RELATED DISEASE

A) INTRODUCTION

The present invention provides hTERT polynucleotides, polypeptides, and
10 antibodies useful for the treatment of human diseases and disease conditions. The recombinant and synthetic hTERT gene products (protein and mRNA) of the invention can be used to create or elevate telomerase activity in a cell, as well as to inhibit telomerase activity in cells in which it is not desired. Thus, inhibiting, activating or otherwise altering a telomerase activity (e.g., telomerase catalytic activity, fidelity,
15 processivity, telomere binding, *etc.*) in a cell can be used to change the proliferative capacity of the cell. For example, reduction of telomerase activity in an immortal cell, such as a malignant tumor cell, can render the cell mortal. Conversely, increasing the telomerase activity in a mortal cell (e.g., most human somatic cells) can increase the proliferative capacity of the cell. For example, expression of hTERT protein in dermal
20 fibroblasts, thereby increasing telomere length, will result in increased fibroblast proliferative capacity; such expression can slow or reverse the age-dependent slowing of wound closure (see, e.g., West, 1994, *Arch. Derm.* 130:87).

Thus, in one aspect, the present invention provides reagents and methods useful for treating diseases and conditions characterized by the presence, absence, or
25 amount of human telomerase activity in a cell and that are susceptible to treatment using the compositions and methods disclosed herein. These diseases include, as described more fully below, cancers, other diseases of cell proliferation (particularly diseases of aging), immunological disorders, infertility (or fertility), and others

B) TREATMENT OF CANCER

The present invention provides methods and compositions for reducing telomerase activity in tumor cells and for treating cancer. Compositions include antisense oligonucleotides, peptides, gene therapy vectors encoding antisense oligonucleotides or activity altering proteins, and anti-hTERT antibodies. Cancer cells (e.g., malignant tumor cells) that express telomerase activity (telomerase-positive cells) can be mortalized by decreasing or inhibiting the endogenous telomerase activity. Moreover, because telomerase levels correlate with disease characteristics such as metastatic potential (e.g., U.S. Patent No. 5,639,613; 5,648,215; 5,489,508; Pandita et al., 1996, *Proc. Am. Ass. Cancer Res.* 37:559), any reduction in telomerase activity could reduce the aggressive nature of a cancer to a more manageable disease state (increasing the efficacy of traditional interventions).

The invention provides compositions and methods useful for treatment of cancers of any of a wide variety of types, including solid tumors and leukemias. Types of cancer that may be treated include (but are not limited to): adenocarcinoma of the breast, prostate, and colon; all forms of bronchogenic carcinoma of the lung; myeloid, melanoma; hepatoma; neuroblastoma; papilloma; apudoma; choristoma; branchioma; malignant carcinoid syndrome; carcinoid heart disease; carcinoma (e.g., Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, in situ, Krebs 2, merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell, and transitional cell), histiocytic disorders; leukemia (e.g., B-cell, mixed-cell, null-cell, T-cell, T-cell chronic, HTLV-II-associated, lymphocytic acute, lymphocytic chronic, mast-cell, and myeloid); histiocytosis malignant; Hodgkin's disease; immunoproliferative small; non-Hodgkin's lymphoma; plasmacytoma; reticuloendotheliosis; melanoma; chondroblastoma; chondroma; chondrosarcoma; fibroma; fibrosarcoma; giant cell tumors; histiocytoma; lipoma; liposarcoma; mesothelioma; myxoma; myxosarcoma; osteoma; osteosarcoma; Ewing's sarcoma; synovioma; adenofibroma; adenolymphoma; carcinosarcoma; chordoma; craniopharyngioma; dysgerminoma; hamartoma; mesenchymoma; mesonephroma; myosarcoma; ameloblastoma; cementoma; odontoma; teratoma; thymoma; trophoblastic tumor; adenocarcinoma; adenoma; cholangioma; cholesteatoma;

cylindroma; cystadenocarcinoma; cystadenoma; granulosa cell tumor;
gynandroblastoma; hepatoma; hidradenoma; islet cell tumor; leydig cell tumor;
papilloma; sertoli cell tumor; theca cell tumor; leiomyoma; leiomyosarcoma;
myoblastoma; myoma; myosarcoma; rhabdomyoma; rhabdomyosarcoma;
5 ependymoma; ganglioneuroma; glioma; medulloblastoma; meningioma;
neurilemmoma; neuroblastoma; neuroepithelioma; neurofibroma; neuroma;
paraganglioma; paraganglioma nonchromaffin; angiokeratoma; angiolymphoid
hyperplasia with eosinophilia; angioma sclerosing; angiomatosis; glomangioma;
hemangioendothelioma; hemangioma; hemangiopericytoma; hemangiosarcoma;
10 lymphangioma; lymphangiomyoma; lymphangiosarcoma; pinealoma; carcinosarcoma;
chondrosarcoma; cystosarcoma phyllodes; fibrosarcoma; hemangiosarcoma;
leiomyosarcoma; leukosarcoma; liposarcoma; lymphangiosarcoma; myosarcoma;
myxosarcoma; ovarian carcinoma; rhabdomyosarcoma; sarcoma (e.g., Ewing's,
experimental, Kaposi's, and mast-cell); neoplasms (e.g., bone, breast, digestive system,
15 colorectal, liver, pancreatic, pituitary, testicular, orbital, head and neck, central nervous
system, acoustic, pelvic, respiratory tract, and urogenital); neurofibromatosis, and
cervical dysplasia). The invention provides compositions and methods useful for
treatment of other conditions in which cells have become immortalized or
hyperproliferative, e.g., by dysregulation (e.g., abnormally high expression) of hTERT,
20 telomerase enzyme, or telomerase activity.

The present invention further provides compositions and methods for
prevention of cancers, including anti-hTERT vaccines, gene therapy vectors that prevent
telomerase activation, and gene therapy vectors that result in specific death of
telomerase-positive cells. In a related aspect, the gene replacement therapy methods
25 described below may be used for "treating" a genetic predilection for cancers.

C) TREATMENT OF OTHER CONDITIONS

The present invention also provides compositions and methods useful
for treatment of diseases and disease conditions (in addition to cancers) characterized
30 by under- or over-expression of telomerase or hTERT gene products. Examples include:
diseases of cell proliferation, diseases resulting from cell senescence (particularly

diseases of aging), immunological disorders, infertility, diseases of immune dysfunction, and others.

Certain diseases of aging are characterized by cell senescence-associated changes due to reduced telomere length (compared to younger cells), resulting from the
5 absence (or much lower levels) of telomerase activity in the cell. Decreased telomere length and decreased replicative capacity contribute to diseases such as those described below. Telomerase activity and telomere length can be increased by, for example, increasing levels of hTERT gene products (protein and mRNA) in the cell. A partial listing of conditions associated with cellular senescence in which hTERT expression can
10 be therapeutic includes Alzheimer's disease, Parkinson's disease, Huntington's disease, and stroke; age-related diseases of the integument such as dermal atrophy, elastolysis and skin wrinkling, sebaceous gland hyperplasia, senile lentigo, graying of hair and hair loss, chronic skin ulcers, and age-related impairment of wound healing; degenerative joint disease; osteoporosis; age-related immune system impairment (e.g., involving
15 cells such as B and T lymphocytes, monocytes, neutrophils, eosinophils, basophils, NK cells and their respective progenitors); age-related diseases of the vascular system including atherosclerosis, calcification, thrombosis, and aneurysms; diabetes, muscle atrophy, respiratory diseases, diseases of the liver and GI tract, metabolic diseases, endocrine diseases (e.g., disorders of the pituitary and adrenal gland), reproductive
20 diseases, and age-related macular degeneration. These diseases and conditions can be treated by increasing the levels of hTERT gene products in the cell to increase telomere length, thereby restoring or imparting greater replicative capacity to the cell. Such methods can be carried out on cells cultured *ex vivo* or cells *in vivo*. In one embodiment, the cells are first treated to activate telomerase and lengthen telomeres,
25 and then treated to inactivate the hTERT gene and telomerase activity. In a preferred embodiment, telomerase activity is generated by a vector of the invention in an embryonic germ or stem cell prior to or during differentiation.

The present invention also provides methods and composition useful for treating infertility. Human germline cells (e.g., spermatogonia cells, their progenitors
30 or descendants) are capable of indefinite proliferation and characterized by high telomerase activity. Abnormal or diminished levels of hTERT gene products can result.

for example, in inadequate or abnormal production of spermatozoa, leading to infertility or disorders of reproduction. Accordingly, "telomerase-based" infertility can be treated using the methods and compositions described herein to increase telomerase levels. Similarly, because inhibition of telomerase may negatively impact spermatogenesis, oogenesis, and sperm and egg viability, the telomerase inhibitory compositions of the invention can have contraceptive effects when used to reduce hTERT gene product levels in germline cells.

Further, the invention provides methods and composition useful for decreasing the proliferative potential of telomerase-positive cells such as activated lymphocytes and hematopoietic stem cells by reducing telomerase activity. Thus, the invention provide means for effecting immunosuppression. Conversely, the methods and reagents of the invention are useful for increasing telomerase activity and proliferative potential in cells, such as stem cells, that express a low level of telomerase or no telomerase prior to therapeutic intervention.

D) MODES OF INTERVENTION

As is clear from the foregoing discussion, modulation of the level of telomerase or telomerase activity of a cell can have a profound effect on the proliferative potential of the cell, and so has great utility in treatment of disease. As is also clear, this modulation may be either a decrease in telomerase activity or an increase in activity. The telomerase modulatory molecules of the invention can act through a number of mechanisms; some of these are described in this and the following subsections to aid the practitioner in selecting therapeutic agents. However, applicants do not intend to be limited to any particular mechanism of action for the novel therapeutic compounds, compositions and methods described herein.

Telomerase activity may be decreased through any of several mechanisms or combinations of mechanisms. One mechanism is the reduction of hTERT gene expression to reduce telomerase activity. This reduction can be at the level of transcription of the hTERT gene into mRNA, processing (e.g., splicing), nuclear transport or stability of mRNA, translation of mRNA to produce hTERT protein, or stability and function of hTERT protein. Another mechanism is interference with one or

more activities of telomerase (e.g., the reverse transcriptase catalytic activity, or the hTR-binding activity) using inhibitory nucleic acids, polypeptides, or other agents (e.g., mimetics, small molecules, drugs and pro-drugs) that can be identified using the methods, or are provided by compositions, disclosed herein. Other mechanisms include sequestration of hTR and/or telomerase associated proteins, and interference with the assembly of the telomerase RNP from its component subunits. In a related mechanism, an hTERT promoter sequence is operably linked to a gene encoding a toxin and introduced into a cell; if or when hTERT transcriptional activators are expressed or activated in the cell, the toxin will be expressed, resulting in specific cell killing.

A related method for reducing the proliferative capacity of a cell involves introducing an hTERT variant with low fidelity (i.e., one with a high, e.g., greater than 1%, error rate) such that aberrant telomeric repeats are synthesized. These aberrant repeats affect telomere protein binding and lead to chromosomal rearrangements and aberrations and/or lead to cell death.

Similarly, telomerase activity may be increased through any of several mechanisms, or a combination of mechanisms. These include increasing the amount of hTERT in a cell. Usually this is carried out by introducing an hTERT polypeptide-encoding polynucleotide into the cell (e.g., a recombinantly produced polypeptide comprising an hTERT DNA sequence operably linked to a promoter, or a stable hTERT mRNA). Alternatively, a catalytically active hTERT polypeptide can itself be introduced into a cell or tissue, e.g., by microinjection or other means known in the art. In other mechanisms, expression from the endogenous hTERT gene or the stability of hTERT gene products in the cell can be increased. Telomerase activity in a cell can also be increased by interfering with the interaction of endogenous telomerase inhibitors and the telomerase RNP, or endogenous hTERT transcription repressors and the hTERT gene; by increasing expression or activity of hTERT transcription activators; and other means apparent to those of skill upon review of this disclosure.

E) INTERVENTION AGENTS

1) TRT PROTEINS & PEPTIDES

In one embodiment, the invention provides telomerase modulatory

polypeptides (i.e., proteins, polypeptides, and peptides) that increase or reduce telomerase activity which can be introduced into a target cell directly (e.g., by injection, liposome-mediated fusion, application of a hydrogel to the tumor [e.g., melanoma] surface, fusion or attachment to herpes virus structural protein VP22, and other means described herein and known in the art). In a second embodiment, telomerase modulatory proteins and peptides of the invention are expressed in a cell by introducing a nucleic acid (e.g., a DNA expression vector or mRNA) encoding the desired protein or peptide into the cell. Expression may be either constitutive or inducible depending on the vector and choice of promoter (*see* discussion below). Messenger RNA preparations encoding hTERT are especially useful when only transient expression (e.g., transient activation of telomerase) is desired. Methods for introduction and expression of nucleic acids into a cell are well known in the art (also, see elsewhere in this specification, e.g., sections on oligonucleotides, gene therapy methods).

In one aspect of the invention, a telomerase modulatory polypeptide that increases telomerase activity in a cell is provided. In one embodiment, the polypeptide is a catalytically active hTERT polypeptide capable of directing the synthesis (in conjunction with an RNA template such as hTR) of human telomeric DNA. This activity can be measured, as discussed above, e.g., using a telomerase activity assay such as a TRAP assay. In one embodiment, the polypeptide is a full-length hTERT protein, having a sequence of, or substantially identical to, the sequence of 1132 residues of SEQUENCE ID No: 2. In another embodiment, the polypeptide is a variant of the hTERT protein of SEQUENCE ID No: 2, such as a fusion polypeptide, derivatized polypeptide, truncated polypeptide, conservatively substituted polypeptide, activity-modified polypeptide, or the like. A fusion or derivatized protein may include a targeting moiety that increases the ability of the polypeptide to traverse a cell membrane or causes the polypeptide to be delivered to a specified cell type (e.g., liver cells or tumor cells) preferentially or cell compartment (e.g., nuclear compartment) preferentially. Examples of targeting moieties include lipid tails, amino acid sequences such as antennapodia peptide or a nuclear localization signal (NLS: e.g., *Xenopus* nucleoplasmin Robbins et al., 1991, *Cell* 64:615). Naturally occurring hTERT protein (e.g., having a sequence of, or substantially identical to, SEQUENCE ID NO: 2) acts in

the cell nucleus. Thus, it is likely that one or more subsequences of SEQUENCE ID NO: 2, such as residues 193-196 (PRRR) and residues 235-240 (PKRPRR) act as a nuclear localization signal. The small regions are likely NLSs based on the observation that many NLSs comprise a 4 residue pattern composed of basic amino acids (K or R), or composed of three basic amino acids (K or R) and H or P; a pattern starting with P and followed within 3 residues by a basic segment containing 3 K or R residues out of 4 residues (see, e.g., Nakai et al., 1992, *Genomics* 14:897). Deletion of one or both of these sequences and/or additional localization sequences is expected to interfere with hTERT transport to the nucleus and/or increase hTERT turnover, and is useful for preventing access of telomerase to its nuclear substrates and decreasing proliferative potential. Moreover, a variant hTERT polypeptide lacking NLS may assemble into an RNP that will not be able to maintain telomere length, because the resulting enzyme cannot enter the nucleus.

The hTERT polypeptides of the invention will typically be associated in the target cell with a telomerase RNA, such as hTR, especially when they are used to increase telomerase activity in a cell. In one embodiment, an introduced hTERT polypeptide associates with an endogenous hTR to form a catalytically active RNP (e.g., an RNP comprising the hTR and a full-length polypeptide having a sequence of SEQUENCE ID NO:2). The RNP so-formed may also associate with other, e.g., telomerase-associated, proteins. In other embodiments, telomerase RNP (containing hTERT protein, hTR and optionally other components) is introduced as a complex to the target cell.

In a related embodiment, an hTERT expression vector is introduced into a cell (or progeny of a cell) into which a telomerase RNA (e.g., hTR) expression vector is simultaneously, subsequently or has been previously introduced. In this embodiment, hTERT protein and telomerase RNA are coexpressed in the cell and assemble to form a telomerase RNP. A preferred telomerase RNA is hTR. An expression vector useful for expression of hTR in a cell is described *supra* (see U.S. Patent 5,583,016). In yet another embodiment, the hTERT polypeptide and hTR RNA (or equivalent) are associated *in vitro* to form a complex, which is then introduced into the target cells, e.g., by liposome mediated transfer.

In another aspect, the invention provides hTRT polypeptides useful for reducing telomerase activity in a cell. As above, these "inhibitory" polypeptides can be introduced directly, or by expression of recombinant nucleic acids in the cell. It will be recognized that peptide mimetics or polypeptides comprising nonstandard amino acids (i.e., other than the 20 amino acids encoded by the genetic code or their normal derivatives) will typically be introduced directly.

In one embodiment, inhibition of telomerase activity results from the sequestration of a component required for accurate telomere elongation. Examples of such components are hTRT and hTR. Thus, administration of a polypeptide that binds hTR, but which does not have telomerase catalytic activity, can reduce endogenous telomerase activity in the cell. In a related embodiment, the hTRT polypeptide may bind a cell component other than hTR, such as one or more telomerase-associated proteins, thereby interfering with telomerase activity in the cell.

In another embodiment, hTRT polypeptides of the invention interfere (e.g., by competition) with the interaction of endogenously expressed hTRT protein and another cellular component required for telomerase function, such as hTR, telomeric DNA, telomerase-associated proteins, telomere-associated proteins, telomeres, cell cycle control proteins, DNA repair enzymes, histone or non-histone chromosomal proteins, or others.

In selecting molecules (e.g., polypeptides) of the invention that affect the interaction of endogenously expressed hTRT protein and other cellular components, one may prefer molecules that include one or more of the conserved motifs of the hTRT protein, as described herein. The evolutionary conservation of these regions indicates the important function in the proper functioning of human telomerase contributed by these motifs, and the motifs are thus generally useful sites for changing hTRT protein function to create variant hTRT proteins of the invention. Thus, variant hTRT polypeptides having mutations in conserved motifs will be particularly useful for some applications of the invention.

In another embodiment, expression of the endogenous hTRT gene is repressed by introduction into the cell of a large amount of hTRT polypeptide (e.g., typically at least about 2-fold more than the endogenous level, more often at least about

10- to about 100-fold) which acts via a feedback loop to inhibit transcription of the hTERT gene, processing of the hTERT pre-mRNA, translation of the hTERT mRNA, or assembly and transport of the telomerase RNP.

5 2) OLIGONUCLEOTIDES

a) ANTISENSE CONSTRUCTS

The invention provides methods and antisense oligonucleotide or polynucleotide reagents which can be used to reduce expression of hTERT gene products *in vitro* or *in vivo*. Administration of the antisense reagents of the invention to a target
10 cell results in reduced telomerase activity, and is particularly useful for treatment of diseases characterized by high telomerase activity (e.g., cancers). Without intending to be limited to any particular mechanism, it is believed that antisense oligonucleotides bind to, and interfere with the translation of, the sense hTERT mRNA. Alternatively, the antisense molecule may render the hTERT mRNA susceptible to nuclease digestion,
15 interfere with transcription, interfere with processing, localization or otherwise with RNA precursors ("pre-mRNA"), repress transcription of mRNA from the hTERT gene, or act through some other mechanism. However, the particular mechanism by which the antisense molecule reduces hTERT expression is not critical.

The antisense polynucleotides of the invention comprise an antisense
20 sequence of at least 7 to 10 to typically 20 or more nucleotides that specifically hybridize to a sequence from mRNA encoding hTERT or mRNA transcribed from the hTERT gene. More often, the antisense polynucleotide of the invention is from about 10 to about 50 nucleotides in length or from about 14 to about 35 nucleotides in length. In other embodiments, antisense polynucleotides are polynucleotides of less than about
25 100 nucleotides or less than about 200 nucleotides. In general, the antisense polynucleotide should be long enough to form a stable duplex but short enough, depending on the mode of delivery, to administer *in vivo*, if desired. The minimum length of a polynucleotide required for specific hybridization to a target sequence depends on several factors, such as G/C content, positioning of mismatched bases (if
30 any), degree of uniqueness of the sequence as compared to the population of target polynucleotides, and chemical nature of the polynucleotide (e.g., methylphosphonate

backbone, peptide nucleic acid, phosphorothioate), among other factors.

Generally, to assure specific hybridization, the antisense sequence is substantially complementary to the target hTERT mRNA sequence. In certain embodiments, the antisense sequence is exactly complementary to the target sequence.

5 The antisense polynucleotides may also include, however, nucleotide substitutions, additions, deletions, transitions, transpositions, or modifications, or other nucleic acid sequences or non-nucleic acid moieties so long as specific binding to the relevant target sequence corresponding to hTERT RNA or its gene is retained as a functional property of the polynucleotide.

10 In one embodiment, the antisense sequence is complementary to relatively accessible sequences of the hTERT mRNA (e.g., relatively devoid of secondary structure). This can be determined by analyzing predicted RNA secondary structures using, for example, the MFOLD program (Genetics Computer Group, Madison WI) and testing *in vitro* or *in vivo* as is known in the art. Examples of
15 oligonucleotides that may be tested in cells for antisense suppression of hTERT function are those capable of hybridizing to (i.e., substantially complementary to) the following positions from SEQUENCE ID NO:1: 40-60; 260-280; 500-520; 770-790; 885-905; 1000-1020; 1300-1320; 1520-1540; 2110-2130; 2295-2315; 2450-2470; 2670-2690; 3080-3110; 3140-3160; and 3690-3710. Another useful method for identifying
20 effective antisense compositions uses combinatorial arrays of oligonucleotides (see, e.g., Milner et al., 1997, *Nature Biotechnology* 15:537).

The invention also provides an antisense polynucleotide that has sequences in addition to the antisense sequence (i.e., in addition to anti-hTERT-sense sequence). In this case, the antisense sequence is contained within a polynucleotide of
25 longer sequence. In another embodiment, the sequence of the polynucleotide consists essentially of, or is, the antisense sequence.

The antisense nucleic acids (DNA, RNA, modified, analogues, and the like) can be made using any suitable method for producing a nucleic acid, such as the chemical synthesis and recombinant methods disclosed herein. In one embodiment, for
30 example, antisense RNA molecules of the invention may be prepared by *de novo* chemical synthesis or by cloning. For example, an antisense RNA that hybridizes to

hTERT mRNA can be made by inserting (ligating) an hTERT DNA sequence (e.g.,
SEQUENCE ID No; 1, or fragment thereof) in reverse orientation operably linked to a
promoter in a vector (e.g., plasmid). Provided that the promoter and, preferably
termination and polyadenylation signals, are properly positioned, the strand of the
5 inserted sequence corresponding to the noncoding strand will be transcribed and act as
an antisense oligonucleotide of the invention.

The antisense oligonucleotides of the invention can be used to inhibit
telomerase activity in cell-free extracts, cells, and animals, including mammals and
humans. For example, the phosphorothioate antisense oligonucleotides:

- 10 A) 5'-GGCATCGCGGGGGTGGCCGGG
B) 5'-CAGCGGGGAGCGCGCGGCATC
C) 5'-CAGCACCTCGCGGTAGTGGCT
D) 5'-GGACACCTGGCGGAAGGAGGG

15 can be used to inhibit telomerase activity. At 10 micromolar concentration each
oligonucleotide, mixtures of oligonucleotides A and B; A, B, C, and D; and A, C, and
D inhibited telomerase activity in 293 cells when treated once per day for seven days.
Inhibition was also observed when an antisense hTR molecule
(5'-GCTCTAGAATGAAGGGTG-3') was used in combination with oligonucleotides
20 A, B, and C; A, B, and D; and A and C. Useful control oligonucleotides in such
experiments include:

- 25 S1) 5'-GCGACGACTGACATTGGCCGG
S2) 5'-GGCTCGAAGTAGCACCGGTGC
S3) 5'-GTGGGAACAGGCCGATGTCCC

To determine the optimum antisense oligonucleotide of the invention for
the particular application of interest, one can perform a scan using antisense
oligonucleotide sets of the invention. One illustrative set is the set of 30-mer
oligonucleotides that span the hTERT mRNA and are offset one from the next by fifteen
30 nucleotides (i.e., ON1 corresponds to positions 1-30 and is
TCCCACGTGCGCAGCAGGACGCAGCGCTGC, ON2 corresponds to positions
16-45 and is GCCGGGGCCAGGGCTTCCCACGTGCGCAGC, and ON3 corresponds
to positions 31-60 and is GGCATCGCGGGGGTGGCCGGGGCCAGGGCT, and so
on to the end of the mRNA). Each member of this set can be tested for inhibitory

activity as disclosed herein. Those oligonucleotides that show inhibitory activity under the conditions of interest then identify a region of interest, and other oligonucleotides of the invention corresponding to the region of interest (i.e., 8-mers, 10-mers, 15-mers, and so on) can be tested to identify the oligonucleotide with the preferred activity for the application.

For general methods relating to antisense polynucleotides, see ANTISENSE RNA AND DNA, (1988), D.A. Melton, Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). See also, Dagle et al., 1991, *Nucleic Acids Research*, 19:1805. For a review of antisense therapy, see, e.g., Uhlmann et al., *Chem. Reviews*, 90:543-584 (1990).

b) TRIPLEX OLIGO- AND POLYNUCLEOTIDES

The present invention provides oligo- and polynucleotides (e.g., DNA, RNA, PNA or the like) that bind to double-stranded or duplex hTERT nucleic acids (e.g., in a folded region of the hTERT RNA or in the hTERT gene), forming a triple helix-containing, or "triplex" nucleic acid. Triple helix formation results in inhibition of hTERT expression by, for example, preventing transcription of the hTERT gene, thus reducing or eliminating telomerase activity in a cell. Without intending to be bound by any particular mechanism, it is believed that triple helix pairing compromises the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or regulatory molecules to occur.

Triplex oligo- and polynucleotides of the invention are constructed using the base-pairing rules of triple helix formation (see, e.g., Cheng et al., 1988, *J. Biol. Chem.* 263: 15110; Ferrin and Camerini-Otero, 1991, *Science* 354:1494; Ramdas et al., 1989, *J. Biol. Chem.* 264:17395; Strobel et al., 1991, *Science* 254:1639; and Rigas et al., 1986, *Proc. Natl. Acad. Sci. U.S.A.* 83: 9591; each of which is incorporated herein by reference) and the hTERT mRNA and/or gene sequence. Typically, the triplex-forming oligonucleotides of the invention comprise a specific sequence of from about 10 to at least about 25 nucleotides or longer "complementary" to a specific sequence in the hTERT RNA or gene (i.e., large enough to form a stable triple helix, but small enough, depending on the mode of delivery, to administer *in vivo*, if desired). In

this context, "complementary" means able to form a stable triple helix. In one embodiment, oligonucleotides are designed to bind specifically to the regulatory regions of the hTERT gene (e.g., the hTERT 5'-flanking sequence, promoters, and enhancers) or to the transcription initiation site, (e.g., between -10 and +10 from the transcription initiation site). For a review of recent therapeutic advances using triplex DNA, see Gee et al., *in* Huber and Carr, 1994, MOLECULAR AND IMMUNOLOGIC APPROACHES, Futura Publishing Co, Mt Kisco NY and Rininsland et al., 1997, *Proc. Natl. Acad. Sci. USA* 94:5854, which are both incorporated herein by reference.

c) RIBOZYMES

The present invention also provides ribozymes useful for inhibition of telomerase activity. The ribozymes of the invention bind and specifically cleave and inactivate hTERT mRNA. Useful ribozymes can comprise 5'- and 3'-terminal sequences complementary to the hTERT mRNA and can be engineered by one of skill on the basis of the hTERT mRNA sequence disclosed herein (see PCT publication WO 93/23572, *supra*). Ribozymes of the invention include those having characteristics of group I intron ribozymes (Cech, 1995, *Biotechnology* 13:323) and others of hammerhead ribozymes (Edgington, 1992, *Biotechnology* 10:256).

Ribozymes of the invention include those having cleavage sites such as GUA, GUU and GUC. Other optimum cleavage sites for ribozyme-mediated inhibition of telomerase activity in accordance with the present invention include those described in PCT publications WO 94/02595 and WO 93/23569, both incorporated herein by reference. Short RNA oligonucleotides between 15 and 20 ribonucleotides in length corresponding to the region of the target hTERT gene containing the cleavage site can be evaluated for secondary structural features that may render the oligonucleotide more desirable. The suitability of cleavage sites may also be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays, or by testing for *in vitro* ribozyme activity in accordance with standard procedures known in the art.

As described by Hu et al., PCT publication WO 94/03596, incorporated herein by reference, antisense and ribozyme functions can be combined in a single

oligonucleotide. Moreover, ribozymes can comprise one or more modified nucleotides or modified linkages between nucleotides, as described above in conjunction with the description of illustrative antisense oligonucleotides of the invention.

5 In one embodiment, the ribozymes of the invention are generated *in vitro* and introduced into a cell or patient. In another embodiment, gene therapy methods are used for expression of ribozymes in a target cell *ex vivo* or *in vivo*.

d) ADMINISTRATION OF OLIGONUCLEOTIDES

10 Typically, the therapeutic methods of the invention involve the administration of an oligonucleotide that functions to inhibit or stimulate telomerase activity under *in vivo* physiological conditions, and is relatively stable under those conditions for a period of time sufficient for a therapeutic effect. As noted above, modified nucleic acids may be useful in imparting such stability, as well as for targeting delivery of the oligonucleotide to the desired tissue, organ, or cell.

15 Oligo- and poly-nucleotides can be delivered directly as a drug in a suitable pharmaceutical formulation, or indirectly by means of introducing a nucleic acid into a cell, including liposomes, immunoliposomes, ballistics, direct uptake into cells, and the like as described herein. For treatment of disease, the oligonucleotides of the invention will be administered to a patient in a therapeutically effective amount. A
20 therapeutically effective amount is an amount sufficient to ameliorate the symptoms of the disease or modulate telomerase activity in the target cell, e.g., as can be measured using a TRAP assay or other suitable assay of telomerase biological function. Methods useful for delivery of oligonucleotides for therapeutic purposes are described in U.S. Patent 5,272,065, incorporated herein by reference. Other details of administration of
25 pharmaceutically active compounds are provided below. In another embodiment, oligo- and poly-nucleotides can be delivered using gene therapy and recombinant DNA expression plasmids of the invention.

3) GENE THERAPY

30 Gene therapy refers to the introduction of an otherwise exogenous polynucleotide which produces a medically useful phenotypic effect upon the

(typically) mammalian cell(s) into which it is transferred. In one aspect, the present invention provides gene therapy methods and compositions for treatment of telomerase-associated conditions. In illustrative embodiments, gene therapy involves introducing into a cell a vector that expresses an hTERT gene product (such as an hTERT protein substantially similar to the hTERT polypeptide having a sequence of SEQUENCE ID NO: 2, e.g., to increase telomerase activity, or an inhibitory hTERT polypeptide to reduce activity), expresses a nucleic acid having an hTERT gene or mRNA sequence (such as an antisense RNA, e.g., to reduce telomerase activity), expresses a polypeptide or polynucleotide that otherwise affects expression of hTERT gene products (e.g., a ribozyme directed to hTERT mRNA to reduce telomerase activity), or replaces or disrupts an endogenous hTERT sequence (e.g., gene replacement and "gene knockout," respectively). Numerous other embodiments will be evident to one of skill upon review of the disclosure herein. In one embodiment, a vector encoding hTR is also introduced. In another embodiment, vectors encoding telomerase-associated proteins are also introduced with or without a vector for hTR.

Vectors useful in hTERT gene therapy can be viral or nonviral, and include those described *supra* in relation to the hTERT expression systems of the invention. It will be understood by those of skill in the art that gene therapy vectors may comprise promoters and other regulatory or processing sequences, such as are described in this disclosure. Usually the vector will comprise a promoter and, optionally, an enhancer (separate from any contained within the promoter sequences) that serve to drive transcription of an oligoribonucleotide, as well as other regulatory elements that provide for episomal maintenance or chromosomal integration and for high-level transcription, if desired. A plasmid useful for gene therapy can comprise other functional elements, such as selectable markers, identification regions, and other sequences. The additional sequences can have roles in conferring stability both outside and within a cell, targeting delivery of hTERT nucleotide sequences (sense or antisense) to a specified organ, tissue, or cell population, mediating entry into a cell, mediating entry into the nucleus of a cell and/or mediating integration within nuclear DNA. For example, aptamer-like DNA structures, or other protein binding moieties sites can be used to mediate binding of a vector to cell surface receptors or to serum proteins that

bind to a receptor thereby increasing the efficiency of DNA transfer into the cell. Other DNA sites and structures can directly or indirectly bind to receptors in the nuclear membrane or to other proteins that go into the nucleus, thereby facilitating nuclear uptake of a vector. Other DNA sequences can directly or indirectly affect the efficiency of integration.

Suitable gene therapy vectors may, or may not, have an origin of replication. For example, it is useful to include an origin of replication in a vector for propagation of the vector prior to administration to a patient. However, the origin of replication can often be removed before administration if the vector is designed to integrate into host chromosomal DNA or bind to host mRNA or DNA. In some situations (e.g., tumor cells) it may not be necessary for the exogenous DNA to integrate stably into the transduced cell, because transient expression may suffice to kill the tumor cells.

As noted, the present invention also provides methods and reagents for gene replacement therapy (i.e., replacement by homologous recombination of an endogenous hTERT gene with a recombinant gene). Vectors specifically designed for integration by homologous recombination may be used. Important factors for optimizing homologous recombination include the degree of sequence identity and length of homology to chromosomal sequences. The specific sequence mediating homologous recombination is also important, because integration occurs much more easily in transcriptionally active DNA. Methods and materials for constructing homologous targeting constructs are described by e.g., Mansour et al., 1988, *Nature* 336: 348; Bradley et al., 1992, *Bio/Technology* 10: 534. See also, U.S. Patent Nos. 5,627,059; 5,487,992; 5,631,153; and 5,464,764. In one embodiment, gene replacement therapy involves altering or replacing all or a portion of the regulatory sequences controlling expression of the hTERT gene that is to be regulated. For example, the hTERT promoter sequences (e.g., such as are found in SEQUENCE ID NO: 6) may be disrupted (to decrease hTERT expression or to abolish a transcriptional control site) or an exogenous promoter (e.g., to increase hTERT expression) substituted.

The invention also provides methods and reagents for hTERT "gene knockout" (i.e., deletion or disruption by homologous recombination of an endogenous

hTERT gene using a recombinantly produced vector). In gene knockout, the targeted sequences can be regulatory sequences (e.g., the hTERT promoter), or RNA or protein coding sequences. The use of homologous recombination to alter expression of endogenous genes is described in detail in U.S. Patent No. 5,272,071 (and the U.S. Patents cited *supra*), WO 91/09955, WO 93/09222, WO 96/29411, WO 95/31560, and WO 91/12650. See also, Moynahan et al., 1996, *Hum. Mol. Genet.* 5:875.

The invention further provides methods for specifically killing telomerase-positive cells, or preventing transformation of telomerase negative cells to a telomerase positive state, using the hTERT gene promoter to regulate expression of a protein toxic to the cell. As shown in Example 14, an hTERT promoter sequence may be operably linked to a reporter gene such that activation of the promoter results in expression of the protein encoded by the reporter gene. If, instead of a reporter protein, the encoded protein is toxic to the cell, activation of the promoter leads to cell morbidity or death. In one embodiment of the present invention, a vector comprising an hTERT promoter operably linked to a gene encoding a toxic protein is introduced into cells, such as human cells, e.g., cells in a human patient, resulting in cell death of cells in which hTERT promoter activating factors are expressed, such as cancer cells. In a related embodiment, the encoded protein is not itself toxic to a cell, but encodes an activity that renders the cell sensitive to an otherwise nontoxic drug. For example, tumors can be treated by introducing an hTERT-promoter-Herpes thymidine kinase (TK) gene fusion construct into tumor cells, and administering gancyclovir or the equivalent (see, e.g., Moolton and Wells, 1990, *J. Nat'l Canc. Inst.* 82:297). The art knows of numerous other suitable toxic or potentially toxic proteins and systems (using promoter sequences other than hTERT) that may be modified and applied in accordance with the present invention by one of skill in the art upon review of this disclosure.

Gene therapy vectors may be introduced into cells or tissues *in vivo*, *in vitro* or *ex vivo*. For *ex vivo* therapy, vectors may be introduced into cells, e.g., stem cells, taken from the patient and clonally propagated for autologous transplant back into the same patient (see, e.g., U.S. Patent Nos. 5,399,493 and 5,437,994, the disclosures of which are herein incorporated by reference). Cells that can be targeted for hTERT gene therapy aimed at increasing the telomerase activity of a target cell include, but are not

limited to, embryonic stem or germ cells, particularly primate or human cells, as noted *supra*, hematopoietic stem cells (AIDS and post-chemotherapy), vascular endothelial cells (cardiac and cerebral vascular disease), skin fibroblasts and basal skin keratinocytes (wound healing and burns), chondrocytes (arthritis), brain astrocytes and microglial cells (Alzheimer's Disease), osteoblasts (osteoporosis), retinal cells (eye diseases), and pancreatic islet cells (Type I diabetes) and any of the cells listed in Table 3, *infra*, as well as any other cell types known to divide.

In one embodiment of the invention, an inducible promoter operably linked to a TRT, such as hTRT, coding sequence (or variant) is used to modulate the proliferative capacity of cells *in vivo* or *in vitro*. In a particular embodiment, for example, insulin-producing pancreatic cells transfected with an hTRT expression vector under the control of an inducible promoter are introduced into a patient. The proliferative capacity of the cells can then be controlled by administration to the patient of the promoter activating agent (e.g., tetracycline) to enable the cells to multiply more than otherwise would have been possible. Cell proliferation can then be terminated, continued, or reinitiated as desired by the treating physician.

4) VACCINES AND ANTIBODIES

Immuogenic peptides or polypeptides having an hTRT sequence can be used to elicit an anti-hTRT immune response in a patient (i.e., act as a vaccine). Exemplary immunogenic hTRT peptides and polypeptides are described *infra* in Examples 6 and 8. An immune response can also be raised by delivery of plasmid vectors encoding the polypeptide of interest (i.e., administration of "naked DNA"). The nucleic acids of interest can be delivered by injection, liposomes, or other means of administration. In one embodiment, immunization modes that elicit in the subject a Class I MHC restricted cytotoxic lymphocyte response against telomerase expressing cells are chosen. Once immunized, the individual or animal will elicit a heightened immune response against cells expressing high levels of telomerase (e.g., malignant cells).

Anti-hTRT antibodies, e.g., murine, human, or humanized monoclonal antibodies may also be administered to a patient (e.g., passive immunization) to effect

an immune response against telomerase-expressing cells.

F) PHARMACEUTICAL COMPOSITIONS

In related aspects, the invention provides pharmaceutical compositions
5 that comprise hTERT oligo- and poly-nucleotides, polypeptides, and antibodies,
agonists, antagonists, or inhibitors, alone or in combination with at least one other
agent, such as a stabilizing compound, diluent, carrier, or another active ingredient or
agent.

The therapeutic agents of the invention may be administered in any
10 sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline,
buffered saline, dextrose, and water. Any of these molecules can be administered to a
patient alone, or in combination with other agents, drugs or hormones, in
pharmaceutical compositions where it is mixed with suitable excipient(s), adjuvants,
and/or pharmaceutically acceptable carriers. In one embodiment of the present
15 invention, the pharmaceutically acceptable carrier is pharmaceutically inert.

Administration of pharmaceutical compositions is accomplished orally
or parenterally. Methods of parenteral delivery include topical, intra-arterial (e.g.,
directly to the tumor), intramuscular, subcutaneous, intramedullary, intrathecal,
intraventricular, intravenous, intraperitoneal, or intranasal administration. In addition
20 to the active ingredients, these pharmaceutical compositions may contain suitable
pharmaceutically acceptable carriers comprising excipients and other compounds that
facilitate processing of the active compounds into preparations which can be used
pharmaceutically. Further details on techniques for formulation and administration
may be found in the latest edition of "REMINGTON'S PHARMACEUTICAL SCIENCES"
25 (Maack Publishing Co, Easton PA).

Pharmaceutical compositions for oral administration can be formulated
using pharmaceutically acceptable carriers well known in the art in dosages suitable for
oral administration. Such carriers enable the pharmaceutical compositions to be
formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries,
30 suspensions, etc., suitable for ingestion by the patient. See PCT publication WO
93/23572.

Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable additional compounds, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers include, but are not limited to sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; and gums including arabic and tragacanth; as well as proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

Dragee cores are provided with suitable coatings such as concentrated sugar solutions, which may also contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound (*i.e.*, dosage).

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with a filler or binders such as lactose or starches, lubricants such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycol with or without stabilizers.

Pharmaceutical formulations for parenteral administration include aqueous solutions of active compounds. For injection, the pharmaceutical compositions of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds may

be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow
5 for the preparation of highly concentrated solutions.

For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

The pharmaceutical compositions of the present invention may be
10 manufactured in a manner similar to that known in the art (e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes).

The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic,
15 lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation may be a lyophilized powder in 1 mM-50 mM histidine, 0.1%-2% sucrose, 2%-7% mannitol at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

20 After pharmaceutical compositions comprising a compound of the invention formulated in an acceptable carrier have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. For administration of human telomerase proteins and nucleic acids, such labeling would include amount, frequency and method of administration.

25 Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. "Therapeutically effective amount" or "pharmacologically effective amount" are well recognized phrases and refer to that amount of an agent effective to produce the intended pharmacological result. Thus, a
30 therapeutically effective amount is an amount sufficient to ameliorate the symptoms of the disease being treated. One useful assay in ascertaining an effective amount for a

given application (e.g., a therapeutically effective amount) is measuring the effect on telomerase activity in a target cell. The amount actually administered will be dependent upon the individual to which treatment is to be applied, and will preferably be an optimized amount such that the desired effect is achieved without significant side-effects. The determination of a therapeutically effective dose is well within the capability of those skilled in the art.

For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays or in any appropriate animal model. The animal model is also used to achieve a desirable concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

A therapeutically effective amount refers to that amount of protein, polypeptide, peptide, antibody, oligo- or polynucleotide, agonist or antagonists which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals (e.g., ED_{50} , the dose therapeutically effective in 50% of the population; and LD_{50} , the dose lethal to 50% of the population). The dose ratio between therapeutic and toxic effects is the therapeutic index, and it can be expressed as the ratio, ED_{50}/LD_{50} . Pharmaceutical compositions which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage is chosen by the individual physician in view of the patient to be treated. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Additional factors which may be taken into account include the severity of the disease state (e.g., tumor size and location; age, weight and gender of the patient; diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy). Long acting pharmaceutical compositions might be administered every 3 to 4

days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation. Guidance as to particular dosages and methods of delivery is provided in the literature (see, US Patent Nos. 4,657,760; 5,206,344; and 5,225,212, herein incorporated by reference). Those skilled in the art will typically employ
5 different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides can be specific to particular cells, conditions, locations, and the like.

VIII. INCREASING PROLIFERATIVE CAPACITY AND PRODUCTION OF 10 IMMORTALIZED CELLS, CELL LINES, AND ANIMALS

As discussed above, most vertebrate cells senesce after a finite number of divisions in culture (e.g., 50 to 100 divisions). Certain variant cells, however, are able to divide indefinitely in culture (e.g., HeLa cells, 293 cells) and, for this reason, are useful for research and industrial applications. Usually these immortal cell lines are
15 derived from spontaneously arising tumors, or by transformation by exposure to radiation or a tumor-inducing virus or chemical. Unfortunately, a limited selection of cell lines, especially human cell lines representing differentiated cell function, is available. Moreover, the immortal cell lines presently available are characterized by chromosomal abnormalities (e.g., aneuploidy, gene rearrangements, or mutations).
20 Further, many long-established cell lines are relatively undifferentiated (e.g., they do not produce highly specialized products of the sort that uniquely characterize particular tissues or organs). Thus, there is a need for new methods of generating immortal cells, especially human cells. One use for immortalized cells is in production of natural proteins and recombinant proteins (e.g., therapeutic polypeptides such as
25 erythropoietin, human growth hormone, insulin, and the like), or antibodies, for which a stable, genetically normal cell line is preferred. For production of some recombinant proteins, specialized cell types may also be preferred (e.g., pancreatic cells for the production of human insulin). Another use for immortalized cells or even mortal cells with increased proliferative capacity (relative to unmodified cells) is for introduction
30 into a patient for gene therapy, or for replacement of diseased or damaged cells or tissue. For example, autologous immune cells containing or expressing a, e.g.,

recombinant hTERT gene or polypeptide of the invention can be used for cell replacement in a patient after aggressive cancer therapy, e.g., whole body irradiation. Another use for immortalized cells is for *ex vivo* production of "artificial" tissues or organs (e.g., skin) for therapeutic use. Another use for such cells is for screening or validation of drugs, such as telomerase-inhibiting drugs, or for use in production of vaccines or biological reagents. Additional uses of the cells of the invention will be apparent to those of skill.

The immortalized cells and cell lines, as well as those of merely increased replicative capacity, of the invention are made by increasing telomerase activity in the cell. Any method disclosed herein for increasing telomerase activity can be used. Thus, in one embodiment, cells are immortalized by increasing the amount of an hTERT polypeptide in the cell. In one embodiment, hTERT levels are increased by introducing an hTERT expression vector into the cell (with stable transfection sometimes preferred). As discussed above, the hTERT coding sequence is usually operably linked to a promoter, which may be inducible or constitutively active in the cell.

In one embodiment, a polynucleotide comprising a sequence encoding a polypeptide of SEQUENCE ID NO: 2, which sequence is operably linked to a promoter (e.g., a constitutively expressed promoter, e.g., a sequence of SEQUENCE ID NO: 6), is introduced into the cell. In one embodiment the polynucleotide comprises a sequence of SEQUENCE ID NO: 1. Preferably the polynucleotide includes polyadenylation and termination signals. In other embodiments, additional elements such as enhancers or others discussed *supra* are included. In an alternative embodiment, the polynucleotide does not include a promoter sequence, such sequence being provided by the target cell endogenous genome following integration (e.g., recombination, e.g., homologous recombination) of the introduced polynucleotide. The polynucleotide may be introduced into the target cell by any method, including any method disclosed herein, such as lipofection, electroporation, virosomes, liposomes, immunoliposomes, polycation:nucleic acid conjugates, naked DNA).

Using the methods of the invention, any vertebrate cell can be caused to have an increased proliferative capacity or even be immortalized and sustained indefinitely in culture. In one embodiment the cells are mammalian, with human cells

preferred for many applications. Examples of human cells that can be immortalized include those listed in Table 3.

It will be recognized that the "diagnostic" assays of the invention described *infra* may be used to identify and characterize the immortalized cells of the invention.

TABLE 3

HUMAN CELLS IN WHICH HTRT EXPRESSION MAY BE INCREASED

- Keratinizing Epithelial Cells**
- 10 keratinocyte of epidermis (differentiating epidermal cell)
 - basal cell of epidermis (stem cell)
 - keratinocyte of fingernails and toenails
 - basal cell of nail bed (stem cell)
 - hair shaft cells
 - 15 medullary, cortical, cuticular; hair-root sheath cells, cuticular, of Huxley's layer, of Henle's layer external; hair matrix cell (stem cell)
- Cells of Wet Stratified Barrier Epithelia**
- 20 surface epithelial cell of stratified squamous epithelium of tongue, oral cavity, esophagus, anal canal, distal urethra, vagina
 - basal cell of these epithelia (stem cell)
 - cell of external corneal epithelium
 - 25 cell of urinary epithelium (lining bladder and urinary ducts)
- Epithelial Cells Specialized for Exocrine Secretion**
- 30 cells of salivary gland
 - mucous cell (secretion rich in polysaccharide)
 - serous cell (secretion rich in glycoprotein enzymes)
 - cell of von Ebner's gland in tongue (secretion to wash over taste buds)
 - cell of mammary gland, secreting milk
 - 35 cell of lacrimal gland, secreting tears
 - cell of ceruminous gland of ear, secreting wax
 - cell of eccrine sweat gland, secreting glycoproteins (dark cell)
 - cell of eccrine sweat gland, secreting small molecules
 - 40 (clear cell)
 - cell of apocrine sweat gland (odoriferous secretion, sex-hormone sensitive)

- cell of gland of Moll in eyelid (specialized sweat gland)
- cell of sebaceous gland, secreting lipid-rich sebum
- cell of Bowman's gland in nose (secretion to wash over olfactory epithelium)
- 5 cell of Brunner's gland in duodenum, secreting alkaline solution of mucus and enzymes
- cell of seminal vesicle, secreting components of seminal fluid, including fructose (as fuel for swimming sperm)
- cell of prostate gland, secreting other components of seminal fluid
- 10 cell of bulbourethral gland, secreting mucus
- cell of Bartholin's gland, secreting vaginal lubricant
- cell of gland of Littre, secreting mucus
- cell of endometrium of uterus, secreting mainly
- 15 carbohydrates
- isolated goblet cell of respiratory and digestive tracts, secreting mucus
- mucous cell of lining of stomach
- zymogenic cell of gastric gland, secreting pepsinogen
- 20 oxyntic cell of gastric gland, secreting HCl
- acinar cell of pancreas, secreting digestive enzymes and bicarbonate
- Paneth cell of small intestine, secreting lysozyme
- type II pneumocyte of lung, secreting surfactant
- 25 Clara cell of lung
-
- Cells specialized for Secretion of Hormones
- cells of anterior pituitary, secreting
- 30 growth hormone, follicle-stimulating hormone, luteinizing hormone, prolactin, adrenocorticotrophic hormone, and thyroid-stimulating hormone,
- cell of intermediate pituitary, secreting melanocyte-stimulating hormone
- cells of posterior pituitary, secreting
- 35 oxytocin, vasopressin
- cells of gut, secreting
- serotonin, endorphin, somatostatin, gastrin, secretin, cholecystokinin, insulin and glucagon
- cells of thyroid gland, secreting
- 40 thyroid hormone, calcitonin
- cells of parathyroid gland, secreting parathyroid hormone, oxyphil cell
- cells of adrenal gland, secreting
- 45 epinephrine, norepinephrine, and steroid hormones; mineralocorticoids glucocorticoids

- cells of gonads, secreting
 - testosterone (Leydig cell of testis)
 - estrogen (theca interna cell of ovarian follicle)
 - progesterone (corpus luteum cell of ruptured ovarian follicle)
- 5 cells of juxtaglomerular apparatus of kidney
 - juxtaglomerular cell (secreting renin)
 - macula densa cell
 - peripolar cell
- 10 mesangial cell
- Epithelial Absorptive Cells in Gut, Exocrine Glands, and Urogenital Tract**
- brush border cell of intestine (with microvilli)
- 15 striated duct cell of exocrine glands
- gall bladder epithelial cell
- brush border cell of proximal tubule of kidney
- distal tubule cell of kidney
- nonciliated cell of ductulus efferens
- 20 epididymal principal cell
- epididymal basal cell
- Cells Specialized for Metabolism and Storage**
- hepatocyte (liver cell)
- 25 fat cells
 - white fat
 - brown fat
 - lipocyte of liver
- 30 **Epithelial Cells Serving Primarily a Barrier Function, Lining the Lung, Gut, Exocrine Glands, and Urogenital Tract**
- type I pneumocyte (lining air space of lung)
- pancreatic duct cell (centroacinar cell)
- 35 nonstriated duct cell of sweat gland, salivary gland, mammary gland
- parietal cell of kidney glomerulus
- podocyte of kidney glomerulus
- cell of thin segment of loop of Henle (in kidney)
- 40 collecting duct cell (in kidney)
- duct cell of seminal vesicle, prostate gland
- Epithelial Cells Lining Closed Internal Body Cavities**
- vascular endothelial cells of blood vessels and lymphatics
- 45 fenestrated
- continuous

- splenic
- synovial cell (lining joint cavities, secreting largely hyaluronic acid)
- serosal cell (lining peritoneal, pleural, and pericardial cavities)
- 5 squamous cell lining perilymphatic space of ear
- cells lining endolymphatic space of ear
- squamous cell
- columnar cells of endolymphatic sac
- 10 with microvilli
- without microvilli
- "dark" cell
- vestibular membrane cell (resembling choroid plexus cell)
- 15 stria vascularis basal cell
- stria vascularis marginal cell
- cell of Claudius
- cell of Boettcher
- choroid plexus cell (secreting cerebrospinal fluid)
- 20 squamous cell of pia-arachnoid
- cells of ciliary epithelium of eye
- pigmented
- nonpigmented
- corneal "endothelial" cell
- 25
- Ciliated Cells with Propulsive Function**
- of respiratory tract
- of oviduct and of endometrium of uterus (in female)
- of rete testis and ductulus efferens (in male)
- 30 of central nervous system (ependymal cell lining brain cavities)
- Cells Specialized for Secretion of Extracellular Matrix epithelial:**
- 35 ameloblast (secreting enamel of tooth)
- planum semilunatum cell of vestibular apparatus of ear (secreting proteoglycan)
- interdental cell of organ of Corti (secreting tectorial "membrane" covering hair cells of organ of Corti)
- 40 nonepithelial (connective tissue)
- fibroblasts (various-of loose connective tissue, of cornea, of tendon, of reticular tissue of bone marrow, etc.)
- pericyte of blood capillary
- 45 nucleus pulposus cell of intervertebral disc
- cementoblast/cementocyte (secreting bonelike cementum of

root of tooth)
 odontoblast/odontocyte (secreting dentin of tooth)
 chondrocytes
 of hyaline cartilage, of fibrocartilage, of elastic
 5 cartilage
 osteoblast/osteocyte
 osteoprogenitor cell (stem cell of osteoblasts)
 hyalocyte of vitreous body of eye
 stellate cell of perilymphatic space of ear
 10

Contractile Cells
 skeletal muscle cells
 red (slow)
 white (fast)
 15 intermediate
 muscle spindle—nuclear bag
 muscle spindle—nuclear chain
 satellite cell (stem cell)
 heart muscle cells
 20 ordinary
 nodal
 Purkinje fiber
 smooth muscle cells
 myoepithelial cells
 25 of iris
 of exocrine glands

Cells of Blood and Immune System
 red blood cell
 30 megakaryocyte
 macrophages
 monocyte
 connective tissue macrophage (various)
 Langerhans cell (in epidermis)
 35 osteoclast (in bone)
 dendritic cell (in lymphoid tissues)
 microglial cell (in central nervous system)
 neutrophil
 eosinophil
 40 basophil
 mast cell
 T lymphocyte
 helper T cell
 suppressor T cell
 45 killer T cell
 B lymphocyte

- IgM
- IgG
- IgA
- IgE
- 5 killer cell
- stem cells for the blood and immune system (various)
- Sensory Transducers**
- photoreceptors
- 10 rod
- cones
- blue sensitive
- green sensitive
- red sensitive
- 15 hearing
- inner hair cell of organ of Corti
- outer hair cell of organ of Corti
- acceleration and gravity
- type I hair cell of vestibular apparatus of ear
- 20 type II hair cell of vestibular apparatus of ear
- taste
- type II taste bud cell
- smell
- olfactory neuron
- 25 basal cell of olfactory epithelium (stem cell for olfactory neurons)
- blood Ph
- carotid body cell
- 30 type I
- type II
- touch
- Merkel cell of epidermis
- primary sensory neurons specialized for touch
- 35 temperature
- primary sensory neurons specialized for temperature
- cold sensitive
- heat sensitive
- pain
- 40 primary sensory neurons specialized for pain
- configurations and forces in musculoskeletal system
- proprioceptive primary sensory neurons
- Autonomic Neurons**
- 45 cholinergic
- adrenergic

peptidergic

Supporting Cells of Sense Organs and of Peripheral Neurons
supporting cells of organ of Corti

- 5 inner pillar cell
- outer pillar cell
- inner phalangeal cell
- outer phalangeal cell
- border cell
- 10 Hensen cell
- supporting cell of vestibular apparatus
- supporting cell of taste bud (type I taste bud cell)
- supporting cell of olfactory epithelium
- Schwann cell
- 15 satellite cell (encapsulating peripheral nerve cell
- bodies)
- enteric glial cell

Neurons and Glial Cells of Central Nervous System

- 20 neurons
- glial cells
- astrocyte
- oligodendrocyte

Lens Cells

- 25 anterior lens epithelial cell
- lens fiber (crystallin-containing cell)

Pigment Cells

- 30 melanocyte
- retinal pigmented epithelial cell

Germ Cells

- oogonium/oocyte
- 35 spermatocyte
- spermatogonium (stem cell for spermatocyte)

Nurse Cells

- ovarian follicle cell
- 40 Sertoli cell (in testis)
- thymus epithelial cell

Stem Cells

- embryonic stem cell
- 45 embryonic germ cell
- adult stem cell

fetal stem cell

IX. DIAGNOSTIC ASSAYS

5 A) INTRODUCTION

1) TRT ASSAYS

The present invention provides a wide variety of assays for TRT, preferably hTRT, and telomerase. These assays provide, *inter alia*, the basis for sensitive, inexpensive, convenient, and widely applicable assays for diagnosis and prognosis of a number of human diseases, of which cancer is an illustrative example. As noted *supra*, hTRT gene products (protein and mRNA) are usually elevated in immortal human cells relative to most normal mortal cells (i.e., telomerase-negative cells and most telomerase-positive normal adult somatic cells). Thus, in one aspect, the invention provides assays useful for detecting or measuring the presence, absence, or quantity of an hTRT gene product in a sample from, or containing, human or other mammalian or eukaryotic cells to characterize the cells as immortal (such as a malignant tumor cell) or mortal (such as most normal somatic cells in adults) or as telomerase positive or negative.

Any condition characterized by the presence or absence of an hTRT gene product (i.e., protein or RNA) may be diagnosed using the methods and materials described herein. These include, as described more fully below, cancers, other diseases of accelerated cell proliferation, immunological disorders, fertility, infertility, and others. Moreover, because the degree to which telomerase activity is elevated in cancer cells is correlated with characteristics of the tumor, such as metastatic potential, monitoring hTRT, mRNA or protein levels can be used to estimate and predict the likely future progression of a tumor.

In one aspect, the diagnostic and prognostic methods of the invention entail determining whether a human TRT gene product is present in a biological sample (e.g., from a patient). In a second aspect, the abundance of hTRT gene product in a biological sample (e.g., from a patient) is determined and compared to the abundance in a control sample (e.g., normal cells or tissues). In a third aspect, the cellular or

intracellular localization of an hTERT gene product is determined in a cell or tissue sample. In a fourth aspect, host (e.g., patient) cells are assayed to identify nucleic acids with sequences characteristic of a heritable propensity for abnormal hTERT gene expression (abnormal quantity, regulation, or product), such as is useful in genetic screening or genetic counseling. In a fifth aspect, the assays of the invention are used to detect the presence of anti-hTERT antibodies (e.g., in patient serum). The methods described below in some detail are indicative of useful assays that can be carried out using the sequences and relationships disclosed herein. However, numerous variations or other applications of these assays will be apparent to those of ordinary skill in the art in view of this disclosure.

It will be recognized that, although the assays below are presented in terms of diagnostic and prognostic methods, they may be used whenever an hTERT gene, gene product, or variant is to be detected, quantified, or characterized. Thus, for example, the "diagnostic" methods described *infra* are useful for assays of hTERT or telomerase during production and purification of hTERT or human telomerase, for characterization of cell lines derived from human cells (e.g., to identify immortal lines), for characterization of cells, non-human animals, plants, fungi, bacteria or other organisms that comprise a human TERT gene or gene product (or fragments thereof).

As used herein, the term "diagnostic" has its usual meaning of identifying the presence or nature of a disease (e.g., cancer), condition (e.g., infertile, activated), or status (e.g., fertile), and the term "prognostic" has its usual meaning of predicting the probable development and/or outcome of a disease or condition. Although these two terms are used in somewhat different ways in a clinical setting, it will be understood that any of the assays or assay formats disclosed below in reference to "diagnosis" are equally suitable for determination of prognosis because it is well established that higher telomerase activity levels are associated with poorer prognoses for cancer patients, and because the present invention provides detection methods specific for hTERT, which is expressed at levels that closely correlate with telomerase activity in a cell.

2) DIAGNOSIS AND PROGNOSIS OF CANCER

The determination of an hTERT gene, mRNA or protein level above normal or standard range is indicative of the presence of telomerase-positive cells, or immortal, of which certain tumor cells are examples. Because certain embryonic and fetal cells, as well as certain adult stem cells, express telomerase, the present invention also provides methods for determining other conditions, such as pregnancy, by the detection or isolation of telomerase positive fetal cells from maternal blood. These values can be used to make, or aid in making, a diagnosis, even when the cells would not have been classified as cancerous or otherwise detected or classified using traditional methods. Thus, the methods of the present invention permit detection or verification of cancerous or other conditions associated with telomerase with increased confidence, and at least in some instances at an earlier stage. The assays of the invention allow discrimination between different classes and grades of human tumors or other cell-proliferative diseases by providing quantitative assays for the hTERT gene and gene products and thereby facilitate the selection of appropriate treatment regimens and accurate diagnoses. Moreover, because levels of telomerase activity can be used to distinguish between benign and malignant tumors (e.g., U.S. Patent No. 5,489,508; Hiyama et al., 1997, *Proc. Am. Ass. Cancer Res.* 38:637), to predict immanence of invasion (e.g., U.S. Patent No. 5,639,613; Yashima et al., 1997, *Proc. Am. Ass. Cancer Res.* 38:326), and to correlate with metastatic potential (e.g., U.S. Patent No. 5,648,215; Pandita et al. 1996, *Proc. Am. Ass. Cancer Res.* 37:559), these assays will be useful for prophylaxis, detection, and treatment of a wide variety of human cancers.

For prognosis of cancers (or other diseases or conditions characterized by elevated telomerase), a prognostic value of hTERT gene product (mRNA or protein) or activity for a particular tumor type, class or grade, is determined as described *infra*. hTERT protein or mRNA levels or telomerase activity in a patient can also be determined (e.g., using the assays disclosed herein) and compared to the prognostic level.

Depending on the assay used, in some cases the abundance of an hTERT gene product in a sample will be considered elevated whenever it is detectable by the assay. Due to the low abundance of hTERT mRNA and protein even in telomerase-

positive cells, and the rarity or non-existence of these gene products in normal or telomerase-negative cells, sensitive assays are required to detect the hTERT gene product if present at all in normal cells. If less sensitive assays are selected, hTERT gene products will be undetectable in healthy tissue but will be detectable in telomerase-positive cancer or other telomerase-positive cells. Typically, the amount of hTERT gene product in an elevated sample is at least about five, frequently at least about ten, more often at least about 50, and very often at least about 100 to 1000 times higher than the levels in telomerase-negative control cells or cells from healthy tissues in an adult, where the percentage of telomerase-positive normal cells is very low.

The diagnostic and prognostic methods of the present invention can be employed with any cell or tissue type of any origin and can be used to detect an immortal or neoplastic cell, or tumor tissue, or cancer, of any origin. Types of cancer that may be detected include, but are not limited to, all those listed *supra* in the discussion of therapeutic applications of hTERT.

The assays of the invention are also useful for monitoring the efficacy of therapeutic intervention in patients being treated with anticancer regimens. Anticancer regimens that can be monitored include all presently approved treatments (including chemotherapy, radiation therapy, and surgery) and also includes treatments to be approved in the future, such as telomerase inhibition or activation therapies as described herein. (See, e.g., See PCT Publication Nos. 96/01835 and 96/40868 and U.S. Patent No. 5,583,016; all of which are incorporated by reference in their entirety).

In another aspect, the assays described below are useful for detecting certain variations in hTERT gene sequence (mutations and heritable hTERT alleles) that are indicative of a predilection for cancers or other conditions associated with abnormal regulation of telomerase activity (infertility, premature aging).

3) DIAGNOSIS OF CONDITIONS OTHER THAN CANCER

In addition to diagnosis of cancers, the assays of the present invention have numerous other applications. The present invention provides reagents and methods/diagnosis of conditions or diseases characterized by under- or over-expression of telomerase or hTERT gene products in cells. In adults, a low level of telomerase activity is normally found in a limited complement of normal human somatic cells, e.g., stem cells, activated lymphocytes and germ cells, and is absent from other somatic cells. Thus, the detection of hTERT or telomerase activity in cells in which it is normally absent or inactive, or detection at abnormal (i.e., higher or lower than normal) levels in cells in which hTERT is normally present at a low level (such as stem cells, activated lymphocytes and germ cells), can be diagnostic of a telomerase-related disease or condition or used to identify or isolate a specific cell type (i.e., to isolate stem cells). Examples of such diseases and conditions include: diseases of cell proliferation, immunological disorders, infertility, diseases of immune cell function, pregnancy, fetal abnormalities, premature aging, and others. Moreover, the assays of the invention are useful for monitoring the effectiveness of therapeutic intervention (including but not limited to drugs that modulate telomerase activity) in a patient or in a cell- or animal-based assay.

In one aspect, the invention provides assays useful for diagnosing infertility. Human germ cells (e.g., spermatogonia cells, their progenitors or descendants) are capable of indefinite proliferation and characterized by high telomerase activity. Abnormal levels or products or diminished levels of hTERT gene products can result in inadequate or abnormal production of spermatozoa, leading to infertility or disorders of reproduction. Accordingly, the invention provides assays (methods and reagents) for diagnosis and treatment of "telomerase-based" reproductive disorders. Similarly, the assays can be used to monitor the efficacy of contraceptives (e.g., male contraceptives) that target or indirectly affect sperm production (and which would reduce hTERT levels or telomerase activity).

In another aspect, the invention provides assays for analysis of telomerase and hTERT levels and function in stem cells, fetal cells, embryonic cells, activated lymphocytes and hematopoietic stem cells. For example, assays for hTERT

gene product detection can be used to monitor immune function generally (e.g., by monitoring the prevalence of activated lymphocytes or abundance of progenitor stem cells), to identify or select or isolate activated lymphocytes or stem cells (based on elevated hTERT levels), and to monitor the efficacy of therapeutic interventions targeting these tissues (e.g., immunosuppressive agents or therapeutic attempt to expand a stem cell population).

The invention also provides assays useful for identification of anti-telomerase and anti-TRT immunoglobulins (found in serum from a patient). The materials and assays described herein can be used to identify patients in which such autoimmune antibodies are found, permitting diagnosis and treatment of the condition associated with the immunoglobulins.

4) MONITORING CELLS IN CULTURE

The assays described herein are also useful for monitoring the expression of hTERT gene products and characterization of hTERT genes in cells *ex vivo* or *in vitro*. Because elevated hTERT levels are characteristic of immortalized cells, the assays of the invention can be used, for example, to screen for, or identify, immortalized cells or to identify an agent capable of mortalizing immortalized cells by inhibiting hTERT expression or function. For example, the assay will be useful for identifying cells immortalized by increased expression of hTERT in the cell, e.g., by the expression of a recombinant hTERT or by increased expression of an endogenously coded hTERT (e.g., by promoter activation).

Similarly, these assays may be used to monitor hTERT expression in transgenic animals or cells (e.g., yeast or human cells containing an hTERT gene). In particular, the effects of certain treatments (e.g., application of known or putative telomerase antagonists) on the hTERT levels in human and nonhuman cells expressing the hTERT of the invention can be used for identifying useful drugs and drug candidates (e.g., telomerase activity-modulating drugs).

B) NORMAL, DIAGNOSTIC, AND PROGNOSTIC VALUES

Assays for the presence or quantity of hTERT gene products may be

carried out and the results interpreted in a variety of ways, depending on the assay format, the nature of the sample being assayed, and the information sought. For example, the steady state abundance of hTERT gene products is so low in most human somatic tissues that they are undetectable by certain assays. Moreover, there is generally no telomerase activity in the cells of these tissues, making verification of activity quite easy. Conversely, hTERT protein and/or hTERT mRNA or telomerase is sufficiently abundant in other telomerase-positive tissues, e.g., malignant tumors, so that the same can be detected using the same assays. Even in those somatic cell types in which low levels of telomerase activity can normally be detected (e.g., stem cells, and certain activated hematopoietic system cells), the levels of hTERT mRNA and telomerase activity are a small fraction (e.g., estimated at about 1% or less) of the levels in immortal cells; thus, immortal and mortal cells may be easily distinguished by the methods of the present invention. It will be appreciated that, when a "less sensitive" assay is used, the mere detection of the hTERT gene product in a biological sample can itself be diagnostic, without the requirement for additional analysis. Moreover, although the assays described below can be made exquisitely sensitive, they may also, if desired, be made less sensitive (e.g., through judicious choice of buffers, wash conditions, numbers of rounds of amplification, reagents, and/or choice of signal amplifiers). Thus, virtually any assay can be designed so that it detects hTERT gene products only in biological samples in which they are present at a particular concentration, e.g. a higher concentration than in healthy or other control tissue. In this case, any detectable level of hTERT mRNA or protein will be considered elevated in cells from post-natal human somatic tissue (other than hematopoietic cells and other stem cells).

In some cases, however, it will be desirable to establish normal or baseline values (or ranges) for hTERT gene product expression levels, particularly when very sensitive assays capable of detecting very low levels of hTERT gene products that may be present in normal somatic cells are used. Normal levels of expression or normal expression products can be determined for any particular population, subpopulation, or group of organisms according to standard methods well known to those of skill in the art and employing the methods and reagents of the invention.

Generally, baseline (normal) levels of hTERT protein or hTERT mRNA are determined by quantitating the amount of hTERT protein and/or mRNA in biological samples (e.g., fluids, cells or tissues) obtained from normal (healthy) subjects, e.g., a human subject. For certain samples and purposes, one may desire to quantitate the amount of hTERT gene product on a per cell, or per tumor cell, basis. To determine the cellularity of a sample, one may measure the level of a constitutively expressed gene product or other gene product expressed at known levels in cells of the type from which the sample was taken. Alternatively, normal values of hTERT protein or hTERT mRNA can be determined by quantitating the amount of hTERT protein/RNA in cells or tissues known to be healthy, which are obtained from the same patient from whom diseased (or possibly diseased) cells are collected or from a healthy individual. Alternatively, baseline levels can be defined in some cases as the level present in non-immortal human somatic cells in culture. It is possible that normal (baseline) values may differ somewhat between different cell types (for example, hTERT mRNA levels will be higher in testis than kidney), or according to the age, sex, or physical condition of a patient. Thus, for example, when an assay is used to determine changes in hTERT levels associated with cancer, the cells used to determine the normal range of hTERT gene product expression can be cells from persons of the same or a different age, depending on the nature of the inquiry. Application of standard statistical methods used in molecular genetics permits determination of baseline levels of expression, as well as permits identification of significant deviations from such baseline levels.

In carrying out the diagnostic and prognostic methods of the invention, as described above, it will sometimes be useful to refer to "diagnostic" and "prognostic values." As used herein, "diagnostic value" refers to a value that is determined for the hTERT gene product detected in a sample which, when compared to a normal (or "baseline") range of the hTERT gene product is indicative of the presence of a disease. The disease may be characterized by high telomerase activity (e.g., cancer), the absence of telomerase activity (e.g., infertility), or some intermediate value. "Prognostic value" refers to an amount of the hTERT gene product detected in a given cell type (e.g., malignant tumor cell) that is consistent with a particular diagnosis and prognosis for the disease (e.g., cancer). The amount (including a zero amount) of the hTERT gene product

detected in a sample is compared to the prognostic value for the cell such that the relative comparison of the values indicates the presence of disease or the likely outcome of the disease (e.g., cancer) progression. In one embodiment, for example, to assess tumor prognosis, data are collected to obtain a statistically significant correlation of hTERT levels with different tumor classes or grades. A predetermined range of hTERT levels is established for the same cell or tissue sample obtained from subjects having known clinical outcomes. A sufficient number of measurements is made to produce a statistically significant value (or range of values) to which a comparison will be made. The predetermined range of hTERT levels or activity for a given cell or tissue sample can then be used to determine a value or range for the level of hTERT gene product that would correlate to favorable (or less unfavorable) prognosis (e.g., a "low level" in the case of cancer). A range corresponding to a "high level" correlated to an (or a more) unfavorable prognosis in the case of cancer can similarly be determined. The level of hTERT gene product from a biological sample (e.g., a patient sample) can then be determined and compared to the low and high ranges and used to predict a clinical outcome.

Although the discussion above refers to cancer for illustration, it will be understood that diagnostic and prognostic values can also be determined for other diseases (e.g., diseases of cell proliferation) and conditions and that, for diseases or conditions other than cancer, a "high" level may be correlated with the desired outcome and a "low" level correlated with an unfavorable outcome. For example, some diseases may be characterized by a deficiency (e.g., low level) of telomerase activity in stem cells, activated lymphocytes, or germline cells. In such cases, "high" levels of hTERT gene products relative to cells of similar age and/or type (e.g., from other patients or other tissues in a particular patient) may be correlated with a favorable outcome.

It will be appreciated that the assay methods do not necessarily require measurement of absolute values of hTERT, unless it is so desired, because relative values are sufficient for many applications of the methods of the present invention. Where quantitation is desirable, the present invention provides reagents such that virtually any known method for quantitating gene products can be used.

The assays of the invention may also be used to evaluate the efficacy of

a particular therapeutic treatment regime in animal studies, in clinical trials, or in monitoring the treatment of an individual patient. In these cases, it may be desirable to establish the baseline for the patient prior to commencing therapy and to repeat the assays one or more times through the course of treatment, usually on a regular basis, to
5 evaluate whether hTERT levels are moving toward the desired endpoint (e.g., reduced expression of hTERT when the assay is for cancer) as a result of the treatment.

One of skill will appreciate that, in addition to the quantity or abundance of hTERT gene products, variant or abnormal expression patterns (e.g., abnormal amounts of RNA splicing variants) or variant or abnormal expression products (e.g.,
10 mutated transcripts, truncated or non-sense polypeptides) may also be identified by comparison to normal expression levels and normal expression products. In these cases determination of "normal" or "baseline" involves identifying healthy organisms and/or tissues (*i.e.* organisms and/or tissues without hTERT expression dysregulation or neoplastic growth) and measuring expression levels of the variant hTERT gene products
15 (e.g., splicing variants), or sequencing or detecting the hTERT gene, mRNA, or reverse transcribed cDNA to obtain or detect typical (normal) sequence variations. Application of standard statistical methods used in molecular genetics permits determination of significant deviations from such baseline levels.

20 C) DETECTION AND QUANTITATION OF TERT GENE PRODUCTS

As has been emphasized herein, hTERT gene products are usually found in most normal somatic cells at extremely low levels. For example, the mRNA encoding hTERT protein is extremely rare or absent in all telomerase-negative cell types studied thus far. In immortal cells, such as 293 cells, hTERT mRNA may be present at
25 only about 100 copies per cell, while normal somatic cells may have as few as one or zero copies per cell. It will thus be apparent that, when highly sensitive assays for hTERT gene products are desired, it will sometimes be advantageous to incorporate signal or target amplification technologies into the assay format. See, for example, Plenat et al., 1997, *Ann. Pathol.* 17:17 (fluoresceinyl-tyramide signal amplification);
30 Zehbe et al., 1997, *J. Pathol.* 150:1553 (catalyzed reporter deposition); other references listed herein (e.g., for bDNA signal amplification, for PCR and other target

amplification formats); and other techniques known in the art.

As noted above, it is often unnecessary to quantitate the hTERT mRNA or protein in the assays disclosed herein, because the detection of an hTERT gene product (under assay conditions in which the product is not detectable in control, e.g., telomerase-negative cells) is in itself sufficient for a diagnosis. As another example, when the levels of product found in a test (e.g., tumor) and control (e.g., healthy cell) samples are directly compared, quantitation may be superfluous.

When desired, however, quantities of hTERT gene product measured in the assays described herein may be described in a variety of ways, depending on the method of measurement and convenience. Thus, normal, diagnostic, prognostic, high or low quantities of hTERT protein/mRNA may be expressed as standard units of weight per quantity of biological sample (e.g., picograms per gram tissue, picograms per 10^{12} cells), as a number of molecules per quantity of biological sample (e.g., transcripts/cell, moles/cell), as units of activity per cell or per other unit quantity, or by similar methods. The quantity of hTERT gene product can also be expressed in relation to the quantity of another molecule: examples include: number of hTERT transcripts in sample/number of 28S rRNA transcripts in sample; nanograms of hTERT protein/nanograms of total protein; and the like.

When measuring hTERT gene products in two (or more) different samples, it will sometimes be useful to have a common basis of comparison for the two samples. For example, when comparing a sample of normal tissue and a sample of cancerous tissue, equal amounts of tissue (by weight, volume, number of cells, etc.) can be compared. Alternatively, equivalents of a marker molecule (e.g., 28S rRNA, hTR, telomerase activity, telomere length, actin) may be used. For example, the amount of hTERT protein in a healthy tissue sample containing 10 picograms of 28S rRNA can be compared to a sample of diseased tissue containing the same amount of 28S rRNA.

It will also be recognized by those of skill that virtually any of the assays described herein can be designed to be quantitative. Typically, a known quantity or source of an hTERT gene product (e.g., produced using the methods and compositions of the invention) is used to calibrate the assay.

In certain embodiments, assay formats are chosen that detect the

presence, absence, or abundance of an hTERT allele or gene product in each cell in a sample (or in a representative sampling). Examples of such formats include those that detect a signal by histology (e.g., immunohistochemistry with signal-enhancing or target-enhancing amplification steps) or fluorescence-activated cell analysis or cell sorting (FACS). These formats are particularly advantageous when dealing with a highly heterogeneous cell population (e.g., containing multiple cell types in which only one or a few types have elevated hTERT levels, or a population of similar cells expressing telomerase at different levels).

D) SAMPLE COLLECTION

The hTERT gene or gene product (i.e., mRNA or polypeptide) is preferably detected and/or quantified in a biological sample. Such samples include, but are not limited to, cells (including whole cells, cell fractions, cell extracts, and cultured cells or cell lines), tissues (including blood, blood cells (e.g., white cells), and tissue samples such as fine needle biopsy samples (e.g., from prostate, breast, thyroid, *etc.*)), body fluids (e.g., urine, sputum, amniotic fluid, blood, peritoneal fluid, pleural fluid, semen) or cells collected therefrom (e.g., bladder cells from urine, lymphocytes from blood), media (from cultured cells or cell lines), and washes (e.g., of bladder and lung). Biological samples may also include sections of tissues such as frozen sections taken for histological purposes. For cancer diagnosis and prognosis, a sample will be obtained from a cancerous or precancerous or suspected cancerous tissue or tumor. It will sometimes be desirable to freeze a biological sample for later analysis (e.g., when monitoring efficacy of drug treatments).

In some cases, the cells or tissues may be fractionated before analysis. For example, in a tissue biopsy from a patient, a cell sorter (e.g., a fluorescence-activated cell sorter) may be used to sort cells according to characteristics such as expression of a surface antigen (e.g., a tumor specific antigen) according to well known methods.

Although the sample is typically taken from a human patient or cell line, the assays can be used to detect hTERT homolog genes or gene products in samples from other animals. Alternatively, hTERT genes and gene products can be assayed in

transgenic animals or organisms expressing a human TRT protein or nucleic acid sequence.

The sample may be pretreated as necessary by dilution in an appropriate buffer solution or concentrated, if desired. Any of a number of standard aqueous buffer solutions, employing one of a variety of buffers, such as phosphate, Tris-buffer, or the like, at physiological pH can be used.

A "biological sample" obtained from a patient can be referred to either as a "biological sample" or a "patient sample." It will be appreciated that analysis of a "patient sample" need not necessarily require removal of cells or tissue from the patient. For example, appropriately labeled hTERT-binding agents (e.g., antibodies or nucleic acids) can be injected into a patient and visualized (when bound to the target) using standard imaging technology (e.g., CAT, NMR, and the like.)

E) NUCLEIC ACID ASSAYS

In one embodiment, this invention provides for methods of detecting and/or quantifying expression of hTERT mRNAs (including splicing or sequence variants and alternative alleles). In an alternative embodiment, the invention provides methods for detecting and analyzing normal or abnormal hTERT genes (or fragments thereof). The form of such qualitative or quantitative assays may include, but is not limited to, amplification-based assays with or without signal amplification, hybridization based assays, and combination amplification-hybridization assays. It will be appreciated by those of skill that the distinction between hybridization and amplification is for convenience only: as illustrated in the examples below, many assay formats involve elements of both hybridization and amplification, so that the categorization is somewhat arbitrary in some cases.

1) PREPARATION OF NUCLEIC ACIDS

In some embodiments, nucleic acid assays are performed with a sample of nucleic acid isolated from the cell, tissue, organism, or cell line to be tested. The nucleic acid (e.g., genomic DNA, RNA or cDNA) may be "isolated" from the sample according to any of a number of methods well known to those of skill in the art. In this context, "isolated" refers to any separation of the species or target to be detected from any other substance in the mixture, but does not necessarily indicate a significant degree of purification of the target. One of skill will appreciate that, where alterations in the copy number of the hTERT gene are to be detected, genomic DNA is the target to be detected. Conversely, where expression levels of a gene or genes are to be detected, RNA is the target to be detected in a nucleic acid-based assay. In one preferred embodiment, the nucleic acid sample is the total mRNA (i.e., poly(A)⁺ RNA) in a biological sample. Methods for isolating nucleic acids are well known to those of skill in the art and are described, for example, Tijssen, P. ed. of LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, PART I. THEORY AND NUCLEIC ACID PREPARATION, Elsevier, N.Y. (1993) Chapt. 3, which is incorporated herein by reference. In one embodiment, the total nucleic acid is isolated from a given sample using an acid guanidinium-phenol-chloroform extraction method and poly(A)⁺ mRNA is isolated by oligo-dT column chromatography or by using (dT)_n magnetic beads (see, e.g., Sambrook et al., and Ausubel et al., *supra*).

In alternative embodiments, it is not necessary to isolate nucleic acids (e.g., total or poly(A)⁺ RNA) from the biological sample prior to carrying out amplification, hybridization or other assays. These embodiments have certain advantages when hTERT RNA is to be measured, because they reduce the possibility of loss of hTERT mRNA during isolation and handling. For example, many amplification techniques such as PCR and RT-PCR defined above can be carried out using permeabilized cells (histological specimens and FACS analyses), whole lysed cells, or crude cell fractions such as certain cell extracts. Preferably, steps are taken to preserve the integrity of the target nucleic acid (e.g., mRNA) if necessary (e.g., addition of RNAase inhibitors). Amplification and hybridization assays can also be carried out *in*

situ, for example, in thin tissue sections from a biopsy sample or from a cell monolayer (e.g., blood cells or disaggregated tissue culture cells). Amplification can also be carried out in an intact whole cell or fixed cells. For example, PCR, RT-PCR, or LCR amplification methods may be carried out, as is well known in the art, *in situ*, e.g., using a polymerase or ligase, a primer or primer(s), and (deoxy)ribonucleoside triphosphates (if a polymerase is employed), and reverse transcriptase and primer (if RNA is to be transcribed and the cDNA is to be detected) on fixed, permeabilized, or microinjected cells to amplify target hTERT RNA or DNA. Cells containing hTERT RNA (e.g., telomerase positive cells) or an hTERT DNA sequence of interest can then be detected. This method is often useful when fluorescently-labeled dNTPs, primers, or other components are used in conjunction with microscopy, FACS analysis or the equivalent.

2) AMPLIFICATION BASED ASSAYS

In one embodiment, the assays of the present invention are amplification-based assays for detection of an hTERT gene or gene product. In an amplification based assay, all or part of an hTERT gene or transcript (e.g., mRNA or cDNA, hereinafter also referred to as "target") is amplified, and the amplification product is then detected directly or indirectly. When there is no underlying gene or gene product to act as a template, no amplification product is produced (e.g., of the expected size), or amplification is non-specific and typically there is no single amplification product. In contrast, when the underlying gene or gene product is present, the target sequence is amplified, providing an indication of the presence and/or quantity of the underlying gene or mRNA. Target amplification-based assays are well known to those of skill in the art.

The present invention provides a wide variety of primers and probes for detecting hTERT genes and gene products. Such primers and probes are sufficiently complementary to the hTERT gene or gene product to hybridize to the target nucleic acid. Primers are typically at least 6 bases in length, usually between about 10 and about 100 bases, typically between about 12 and about 50 bases, and often between about 14 and about 25 bases in length. One of skill, having reviewed the present disclosure, will be able, using routine methods, to select primers to amplify all, or any

portion, of the hTRT gene or gene product, or to distinguish between variant gene products, hTRT alleles, and the like. Table 2 lists illustrative primers useful for PCR amplification of the hTRT, or specific hTRT gene products or regions. As is known in the art, single oligomers (e.g., U.S. Pat. No. 5,545,522), nested sets of oligomers, or even a degenerate pool of oligomers may be employed for amplification, e.g., as illustrated by the amplification of the *Tetrahymena* TRT cDNA as described *infra*.

The invention provides a variety of methods for amplifying and detecting an hTRT gene or gene product, including the polymerase chain reaction (including all variants, e.g., reverse-transcriptase-PCR; the Sunrise Amplification System (Oncor, Inc, Gaithersburg MD); and numerous others known in the art). In one illustrative embodiment, PCR amplification is carried out in a 50 μ l solution containing the nucleic acid sample (e.g., cDNA obtained through reverse transcription of hTRT RNA), 100 μ M in each dNTP (dATP, dCTP, dGTP and dTTP; Pharmacia LKB Biotechnology, NJ), the hTRT-specific PCR primer(s), 1 unit/ Taq polymerase (Perkin Elmer, Norwalk CT), 1x PCR buffer (50 mM KCl, 10 mM Tris, pH 8.3 at room temperature, 1.5 mM MgCl₂, 0.01% gelatin) with the amplification run for about 30 cycles at 94° for 45 sec, 55° for 45 sec and 72° for 90 sec. However, as will be appreciated, numerous variations may be made to optimize the PCR amplification for any particular reaction.

Other suitable target amplification methods include the ligase chain reaction (LCR; e.g., Wu and Wallace, 1989, *Genomics* 4:560; Landegren *et al.*, 1988, *Science*, 241: 1077, Barany, 1991, *Proc. Natl. Acad. Sci. USA* 88:189 and Barringer *et al.*, 1990, *Gene*, 89: 117); strand displacement amplification (SDA; e.g., Walker *et al.*, 1992, *Proc. Natl. Acad. Sci. U.S.A.* 89:392-396); transcription amplification (e.g., Kwoh *et al.*, 1989, *Proc. Natl. Acad. Sci. USA*, 86: 1173); self-sustained sequence replication (3SR; e.g., Fahy *et al.*, 1992, *PCR Methods Appl.* 1:25, Guatelli *et al.*, 1990, *Proc. Nat. Acad. Sci. USA*, 87: 1874); the nucleic acid sequence based amplification (NASBA, Cangene, Mississauga, Ontario; e.g., Compton, 1991, *Nature* 350:91); the transcription-based amplification system (TAS); and the self-sustained sequence replication system (SSR). Each of the aforementioned publications is incorporated herein by reference. One useful variant of PCR is PCR ELISA (e.g., Boehringer

Mannheim Cat. No. 1 636 111) in which digoxigenin-dUTP is incorporated into the PCR product. The PCR reaction mixture is denatured and hybridized with a biotin-labeled oligonucleotide designed to anneal to an internal sequence of the PCR product. The hybridization products are immobilized on streptavidin coated plates and detected using anti-digoxigenin antibodies. Examples of techniques sufficient to direct persons of skill through *in vitro* amplification methods are found in PCR TECHNOLOGY: PRINCIPLES AND APPLICATIONS FOR DNA AMPLIFICATION, H. Erlich, Ed. Freeman Press, New York, NY (1992); PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS, eds. Innis, Gelfand, Snisky, and White, Academic Press, San Diego, CA (1990); Matila et al., 1991, *Nucleic Acids Res.* 19: 4967; Eckert and Kunkel, (1991) PCR METHODS AND APPLICATIONS 1: 17; PCR, eds. McPherson, Quirk, and Taylor, IRL Press, Oxford; U.S. Patent Nos. 4,683,195, 4,683,202, and 4,965,188; Barringer et al., 1990, *Gene*, 89:117; Lomell et al., 1989, *J. Clin. Chem.*, 35:1826, each of which is incorporated herein for all purposes.

Amplified products may be directly analyzed, e.g., by size as determined by gel electrophoresis; by hybridization to a target nucleic acid immobilized on a solid support such as a bead, membrane, slide, or chip; by sequencing; immunologically, e.g., by PCR-ELISA, by detection of a fluorescent, phosphorescent, or radioactive signal; or by any of a variety of other well-known means. For example, an illustrative example of a detection method uses PCR primers augmented with hairpin loops linked to fluorescein and a benzoic acid derivative that serves as a quencher, such that fluorescence is emitted only when the primers unfold to bind their targets and replication occurs.

Because hTERT mRNA is typically expressed as an extremely rare transcript, present at very low levels even in telomerase positive cells, it is often desirable to optimize or increase the signal resulting from the amplification step. One way to do this is to increase the number of cycles of amplification. For example, although 20-25 cycles are adequate for amplification of most mRNAs using the polymerase chain reaction under standard reaction conditions, detection of hTERT mRNA in many samples can require as many as 30 to 35 cycles of amplification, depending on detection format and efficiency of amplification. It will be recognized

that judicious choice of the amplification conditions including the number of amplification cycles can be used to design an assay that results in an amplification product only when there is a threshold amount of target in the test sample (i.e., so that only samples with a high level of hTERT mRNA give a "positive" result). In addition, methods are known to increase signal produced by amplification of the target sequence. Methods for augmenting the ability to detect the amplified target include signal amplification system such as: branched DNA signal amplification (e.g., U.S. Pat. No. 5,124,246; Urdea, 1994, *Bio/Tech.* 12:926); tyramide signal amplification (TSA) system (Du Pont); catalytic signal amplification (CSA; Dako); Q Beta Replicase systems (Tyagi et al., 1996, *Proc. Nat. Acad. Sci. USA*, 93: 5395); or the like.

One of skill in the art will appreciate that whatever amplification method is used, a variety of quantitative methods known in the art can be used if quantitation is desired. For example, when desired, two or more polynucleotides can be co-amplified in a single sample. This method can be used as a convenient method of quantitating the amount of hTERT mRNA in a sample, because the reverse transcription and amplification reactions are carried out in the same reaction for a target and control polynucleotide. The co-amplification of the control polynucleotide (usually present at a known concentration or copy number) can be used for normalization to the cell number in the sample as compared to the amount of hTERT in the sample. Suitable control polynucleotides for co-amplification reactions include DNA, RNA expressed from housekeeping genes, constitutively expressed genes, and *in vitro* synthesized RNAs or DNAs added to the reaction mixture. Endogenous control polynucleotides are those that are already present in the sample, while exogenous control polynucleotides are added to a sample, creating a "spiked" reaction. Illustrative control RNAs include β -actin RNA, GAPDH RNA, snRNAs, hTR, and endogenously expressed 28S rRNA (see Khan et al., 1992, *Neurosci. Lett.* 147:114). Exogenous control polynucleotides include a synthetic AW106 cRNA, which may be synthesized as a sense strand from pAW106 by T7 polymerase. It will be appreciated that for the co-amplification method to be useful for quantitation, the control and target polynucleotides must typically both be amplified in a linear range. Detailed protocols for quantitative PCR may be found in PCR PROTOCOLS. A GUIDE TO METHODS AND APPLICATIONS. Innis et al., Academic

Press, Inc. N.Y., (1990) and Ausubel et al., *supra* (Unit 15) and Diaco, R. (1995) *Practical Considerations for the Design of Quantitative PCR Assays*, in PCR STRATEGIES, pg. 84-108, Innis et al. eds, Academic Press, New York.

Depending on the sequence of the endogenous or exogenous standard, different primer sets may be used for the co-amplification reaction. In one method, called competitive amplification, quantitative PCR involves simultaneously co-amplifying a known quantity of a control sequence using the same primers used for amplification of the target nucleic acid (one pair of 2 primers). In an alternative embodiment, known as non-competitive competition, the control sequence and the target sequence (e.g., hTERT cDNA) are amplified using different primers (i.e., 2 pairs of 2 primers). In another alternative embodiment, called semi-competitive amplification, three primers are used, one of which is hTERT-specific, one of which is control specific, and one of which is capable of annealing to both the target and control sequences. Semi-competitive amplification is described in U.S. Patent No. 5,629,154, which is incorporated herein by reference.

3) HYBRIDIZATION-BASED ASSAYS

a) GENERALLY

A variety of methods for specific DNA and RNA measurement using nucleic acid hybridization techniques are known to those of skill in the art (see Sambrook et al., *supra*). Hybridization based assays refer to assays in which a probe nucleic acid is hybridized to a target nucleic acid. Usually the nucleic acid hybridization probes of the invention are entirely or substantially identical to a contiguous sequence of the hTERT gene or RNA sequence. Preferably, nucleic acid probes are at least about 10 bases, often at least about 20 bases, and sometimes at least about 200 bases or more in length. Methods of selecting nucleic acid probe sequences for use in nucleic acid hybridization are discussed in Sambrook et al., *supra*. In some formats, at least one of the target and probe is immobilized. The immobilized nucleic acid may be DNA, RNA, or another oligo- or poly-nucleotide, and may comprise natural or non-naturally occurring nucleotides, nucleotide analogs, or backbones. Such assays may be in any of several formats including: Southern, Northern, dot and slot

blots, high-density polynucleotide or oligonucleotide arrays (e.g., GeneChips™ Affymetrix), dip sticks, pins, chips, or beads. All of these techniques are well known in the art and are the basis of many commercially available diagnostic kits. Hybridization techniques are generally described in Hames et al., ed., NUCLEIC ACID HYBRIDIZATION, A PRACTICAL APPROACH IRL Press, (1985); Gall and Pardue *Proc. Natl. Acad. Sci., U.S.A.*, 63: 378-383 (1969); and John et al., *Nature*, 223: 582-587 (1969).

A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, one common format is direct hybridization, in which a target nucleic acid is hybridized to a labeled, complementary probe. Typically, labeled nucleic acids are used for hybridization, with the label providing the detectable signal. One method for evaluating the presence, absence, or quantity of hTERT mRNA is carrying out a Northern transfer of RNA from a sample and hybridization of a labeled hTERT specific nucleic acid probe, as illustrated in Example 2. As was noted *supra*, hTERT mRNA, when present at all, is present in very low quantities in most cells.

Therefore, when Northern hybridization is used, it will often be desirable to use an amplification step (or, alternatively, large amounts of starting RNA). A useful method for evaluating the presence, absence, or quantity of DNA encoding hTERT proteins in a sample involves a Southern transfer of DNA from a sample and hybridization of a labeled hTERT specific nucleic acid probe.

Other common hybridization formats include sandwich assays and competition or displacement assays. Sandwich assays are commercially useful hybridization assays for detecting or isolating nucleic acid sequences. Such assays utilize a "capture" nucleic acid covalently immobilized to a solid support and a labeled "signal" nucleic acid in solution. The biological or clinical sample will provide the target nucleic acid. The "capture" nucleic acid and "signal" nucleic acid probe hybridize with the target nucleic acid to form a "sandwich" hybridization complex. To be effective, the signal nucleic acid cannot hybridize with the capture nucleic acid.

b) CHIP-BASED AND SLIDE-BASED ASSAYS

The present invention also provides probe-based hybridization assays for hTERT gene products employing arrays of immobilized oligonucleotide or

polynucleotides to which an hTERT nucleic acid can hybridize (i.e., to some, but usually not all or even most, of the immobilized oligo- or poly-nucleotides). High density oligonucleotide arrays or polynucleotide arrays provide a means for efficiently detecting the presence and characteristics (e.g., sequence) of a target nucleic acid (e.g., hTERT gene, mRNA, or cDNA). Techniques are known for producing arrays containing thousands of oligonucleotides complementary to defined sequences, at defined locations on a surface using photolithographic techniques for synthesis *in situ* (see, e.g., U.S. Patent Nos. 5,578,832; 5,556,752; and 5,510,270; Fodor et al., 1991, *Science* 251:767; Pease et al., 1994, *Proc. Natl. Acad. Sci. USA* 91:5022; and Lockhart et al., 1996, *Nature Biotech* 14:1675) or other methods for rapid synthesis and deposition of defined oligonucleotides (Blanchard et al., 1996, *Biosensors & Bioelectronics* 11:687). When these methods are used, oligonucleotides (e.g., 20-mers) of known sequence are synthesized directly on a surface such as a derivatized glass slide. Usually, the array produced is redundant, having several oligonucleotide probes on the chip specific for the hTERT polynucleotide to be detected.

Combinations of oligonucleotide probes can be designed to detect alternatively spliced mRNAs, or to identify which of various hTERT alleles is expressed in a particular sample.

In one illustrative embodiment, cDNA prepared by reverse transcription of total RNA from a test cell is amplified (e.g., using PCR). Typically the amplification product is labeled, e.g., by incorporation of a fluorescently labeled dNTP. The labeled cDNAs are then hybridized to a chip comprising oligonucleotide probes complementary to various subsequences of the hTERT gene. The positions of hybridization are determined (e.g., in accordance with the general methods of Shalon et al., 1996, *Genome Research* 6:639 or Schena et al., 1996, *Genome Res.* 6:639), and sequence (or other information) deduced from the hybridization pattern, by means well known in the art.

In one embodiment, two cDNA samples, each labeled with a different fluorescent group, are hybridized to the same chip. The ratio of the hybridization of each labeled sample to sites complementary to the hTERT gene are then assayed. If both samples contain the same amount of hTERT mRNA, the ratio of the two fluors will be

1:1 (it will be appreciated that the signal from the fluors may need to be adjusted to account for any difference in the molar sensitivity of the fluors). In contrast, if one sample is from a healthy (or control) tissue and the second sample is from a cancerous tissue the fluor used in the second sample will predominate.

5

c) IN SITU HYBRIDIZATION

An alternative means for detecting expression of a gene encoding an hTERT protein is *in situ* hybridization. *In situ* hybridization assays are well known and are generally described in Angerer et al., METHODS ENZYMOL., 152: 649-660 (1987) and Ausubel et al., *supra*. In an *in situ* hybridization assay, cells or tissue specimens are fixed to a solid support, typically in a permeabilized state, typically on a glass slide. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of labeled nucleic acid probes (e.g., ³⁵S-labeled riboprobes, fluorescently labeled probes) completely or substantially complementary to hTERT. Free probe is removed by washing and/or nuclease digestion, and bound probe is visualized directly on the slide by autoradiography or an appropriate imaging techniques, as is known in the art.

10

15

4) SPECIFIC DETECTION OF VARIANTS

20

As noted *supra* and illustrated in the Examples (e.g., Example 9), amplification primers or probes can be selected to provide amplification products that span specific deletions, truncations, and insertions, thereby facilitating the detection of specific variants or abnormalities in the hTERT mRNA.

25

One example of an hTERT variant gene product that may be detected is an hTERT RNA such as a product (SEQUENCE ID NO: 4) described *supra* and in Example 9. The biological function, if any, of the $\Delta 182$ variant(s) is not known; however, the truncated hTERT protein putatively encoded by the variant may be involved in regulation of telomerase activity, e.g., by assembling a non-functional telomerase RNP that titrates telomerase components. Alternatively, negative regulation of telomerase activity could be accomplished by directing hTERT pre-mRNA (nascent mRNA) processing in a manner leading to elimination of the full length mRNA and

30

reducing hTERT mRNA levels and increasing $\Delta 182$ hTERT RNA levels. For these and other reasons, the ability to detect $\Delta 182$ variants is useful. In addition, it will sometimes be desirable, in samples in which two species of hTERT RNA are present (such as a $\Delta 182$ hTERT RNA and hTERT RNA encoding the full-length hTERT protein) to compare their relative and/or absolute abundance.

The invention provides a variety of methods for detection of $\Delta 182$ variants. For example, amplification using primer pairs spanning the 182 basepair deletion will result in different sized products corresponding to the deleted and undeleted hTERT RNAs, if both are present, which can be distinguished on the basis of size (e.g., by gel electrophoresis). Examples of primer pairs useful for amplifying the region spanning the 182 bp deletion include TCP1.14 and TCP1.15 (primer set 1), or TCP1.25 and hTCP6 (primer set 2) (see Table 2). These primer pairs can be used individually or in a nested PCR experiment where primer set 1 is used first. It will also be apparent to one of skill that hybridization methods (e.g., Northern hybridization) or RNase protection assays using an hTERT nucleic acid probe of the invention can be used to detect and distinguish hTERT RNA variants.

Another suitable method entails PCR amplification (or the equivalent) using three primers. Analogous to the semi-competitive quantitative PCR method described in greater detail *supra*, one primer is specific to each of the hTERT RNA species (e.g., as illustrated in Table 4) and one primer is complementary to both species (e.g., TCP1.25 (2270-2288)). An example of a primer specific to SEQUENCE ID NO: 1 is one that anneals within the 182 nucleotide sequence (i.e., nucleotides 2345 to 2526 of SEQUENCE ID NO: 1), e.g., TCP1.73 (2465-2445). For example, a primer specific to SEQUENCE ID NO: 4 (a $\Delta 182$ variant) is one that anneals at nucleotides 2358 to 2339 of SEQUENCE ID NO: 4 (i.e., the site corresponding to the 182 nucleotide insertion in SEQUENCE ID NO: 1). The absolute abundance of the $\Delta 182$ hTERT mRNA species or its relative abundance compared to the species encoding the full-length hTERT protein can be analyzed for correlation to cell state (e.g., capacity for indefinite proliferation). It will be appreciated that numerous other primers or amplification or detection methods can be selected based on the present disclosure.

TABLE 4
ILLUSTRATIVE PRIMERS

Δ182 species (e.g., SEQUENCE ID NO. 4) specific primer:

5'-GGCACTGGACGTAGGACGTG-3

5 hTRT (SEQUENCE ID NO. 1) specific primer (TCP1.73):

5'-CACTGCTGGCCTCATTGAGG-3

Common (forward) primer (TCP1.25):

5'-TACTGCGTTCGTCGGTATG-3'

10 Other variant hTRT genes or gene products that can be detected include those characterized by premature stop codons, deletions, substitutions or insertions. Deletions can be detected by the decreased size of the gene, mRNA transcript, or cDNA. Similarly, insertions can be detected by the increased size of the gene, mRNA transcript, or cDNA. Insertions and deletions could also cause shifts in the reading

15 frame that lead to premature stop codons or longer open reading frames. Substitutions, deletions, and insertions can also be detected by probe hybridization. Alterations can also be detected by observing changes in the size of the variant hTRT polypeptide (e.g., by Western analysis) or by hybridization or specific amplification as appropriate. Alternatively, mutations can be determined by sequencing of the gene or gene product

20 according to standard methods. In addition, and as noted above, amplification assays and hybridization probes can be selected to target particular abnormalities specifically. For example, nucleic acid probes or amplification primers can be selected that specifically hybridize to or amplify, respectively, the region encompassing the deletion, substitution, or insertion. Where the hTRT gene harbors such a mutation, the probe

25 will either (1) fail to hybridize or the amplification reaction will fail to provide specific amplification or cause a change in the size of the amplification product or hybridization signal; or (2) the probe or amplification reaction encompasses the entire deletion or either end of the deletion (deletion junction); or (3) similarly, probes and amplification primers can be selected that specifically target point mutations or insertions.

30

5) DETECTION OF MUTANT hTERT ALLELES

Mutations in the hTERT gene can be responsible for disease initiation or can contribute to a disease condition. Alterations of the genomic DNA of hTERT can affect levels of gene transcription, change amino acid residues in the hTERT protein, cause truncated hTERT polypeptides to be produced, alter pre-mRNA processing pathways (which can alter hTERT mRNA levels), and cause other consequences as well.

Alterations of genomic DNA in non-hTERT loci can also affect expression of hTERT or telomerase by altering the enzymes or cellular processes that are responsible for regulating hTERT, hTR, and telomerase-associated protein expression and processing and RNP assembly and transport. Alterations which affect hTERT expression, processing, or RNP assembly could be important for cancer progression, for diseases of aging, for DNA damage diseases, and others.

Detection of mutations in hTERT mRNA or its gene and gene control elements can be accomplished in accordance with the methods herein in multiple ways.

Illustrative examples include the following: A technique termed primer screening can be employed: PCR primers are designed whose 3' termini anneal to nucleotides in a sample DNA (or RNA) that are possibly mutated. If the DNA (or RNA) is amplified by the primers, then the 3' termini matched the nucleotides in the gene; if the DNA is not amplified, then one or both termini did not match the nucleotides in the gene, indicating a mutation was present. Similar primer design can be used to assay for point mutations using the Ligase Chain Reaction (LCR, described *supra*). Restriction fragment length polymorphism, RFLP (Pourzand, C., Cerutti, P. (1993) *Mutat. Res* 288: 113-121), is another technique that can be applied in the present method. A Southern blot of human genomic DNA digested with various restriction enzymes is probed with an hTERT specific probe. Differences in the fragment number or sizes between the sample and a control indicate an alteration of the experimental sample, usually an insertion or deletion. Single strand conformation polymorphism, SSCP (Orrita, M., et al. (1989) *PNAS USA* 86:2766-70), is another technique that can be applied in the present method. SSCP is based on the differential migration of denatured wild-type and mutant single-stranded DNA (usually generated by PCR). Single-stranded DNA will take on a three-dimensional conformation that is

sequence-specific. Sequence differences as small as a single base change can result in a mobility shift on a nondenaturing gel. SSCP is one of the most widely used mutation screening methods because of its simplicity. Denaturing Gradient Gel Electrophoresis, DGGE (Myers, R. M., Maniatis, T. and Lerman, L., (1987) *Methods in Enzymology*, 155: 501-527), is another technique that can be applied in the present method. DGGE identifies mutations based on the melting behavior of double-stranded DNA. Specialized denaturing electrophoresis equipment is utilized to observe the melting profile of experimental and control DNAs: a DNA containing a mutation will have a different mobility compared to the control in these gel systems. The examples discussed illustrate commonly employed methodology; many other techniques exist which are known by those skilled in the art and can be applied in accordance with the teachings herein.

F. KARYOTYPE ANALYSIS

The present invention further provides methods and reagents for karyotype or other chromosomal analysis using hTERT-sequence probes and/or detecting or locating hTERT gene sequences in chromosomes from a human patient, human cell line, or non-human cell. In one embodiment, amplification (i.e., change in copy number), deletion (i.e., partial deletion), insertion, substitution, or changes in the chromosomal location (e.g., translocation) of an hTERT gene may be correlated with the presence of a pathological condition or a predisposition to developing a pathological condition (e.g., cancer).

It has been determined by the present inventors that, in normal human cells, the hTERT gene maps close to the telomere of chromosome 5p (see Example 5, *infra*). The closest STS marker is D5S678 (see Figure 8). The location can be used to identify markers that are closely linked to the hTERT gene. The markers can be used to identify YACs, STSs, cosmids, BACs, lambda or P1 phage, or other clones which contain hTERT genomic sequences or control elements. The markers or the gene location can be used to scan human tissue samples for alterations in the normal hTERT gene location, organization or sequence that is associated with the occurrence of a type of cancer or disease. This information can be used in a diagnostic or prognostic manner

for the disease or cancer involved. Moreover, the nature of any alterations to the hTERT gene can be informative as to the nature by which cells become immortal. For instance, a translocation event could indicate that activation of hTERT expression occurs in some cases by replacing the hTERT promoter with another promoter which directs hTERT transcription in an inappropriate manner. Methods and reagents of the invention of this type can be used to inhibit hTERT activation. The location may also be useful for determining the nature of hTERT gene repression in normal somatic cells, for instance, whether the location is part of non-expressing heterochromatin. Nuclease hypersensitivity assays for distinguishing heterochromatin and euchromatin are described, for example, in Wu et al., 1979, *Cell* 16:797; Groudine and Weintraub, 1982, *Cell* 30:131 Gross and Garrard, 1988, *Ann. Rev. Biochem.* 57:159.

In one embodiment, alterations to the hTERT gene are identified by karyotype analysis, using any of a variety of methods known in the art. One useful technique is *in situ* hybridization (ISH). Typically, when *in situ* hybridization techniques are used for karyotype analysis, a detectable or detectably-labeled probe is hybridized to a chromosomal sample *in situ* to locate an hTERT gene sequence. Generally, ISH comprises one or more of the following steps: (1) fixation of the tissue, cell or other biological structure to be analyzed; (2) prehybridization treatment of the biological structure to increase accessibility of target DNA (e.g., denaturation with heat or alkali), and to reduce nonspecific binding (e.g., by blocking the hybridization capacity of repetitive sequences, e.g., using human genomic DNA); (3) hybridization of one or more nucleic acid probes (e.g., conventional nucleic acids, PNAs, or probes containing other nucleic acid analogs) to the nucleic acid in the biological structure or tissue; (4) posthybridization washes to remove nucleic acid fragments not bound in the hybridization; and, (5) detection of the hybridized nucleic acid fragments. The reagents used in each of these steps and conditions for their use vary depending on the particular application. It will be appreciated that these steps can be modified in a variety of ways well known to those of skill in the art.

In one embodiment of ISH, the hTERT probe is labeled with a fluorescent label (fluorescent *in situ* hybridization; "FISH"). Typically, it is desirable to use dual color fluorescent *in situ* hybridization, in which two probes are utilized, each labeled by

a different fluorescent dye. A test probe that hybridizes to the hTERT sequence of interest is labeled with one dye, and a control probe that hybridizes to a different region is labeled with a second dye. A nucleic acid that hybridizes to a stable portion of the chromosome of interest, such as the centromere region, can be used as the control probe. In this way, one can account for differences between efficiency of hybridization from sample to sample.

The ISH methods for detecting chromosomal abnormalities (e.g., FISH) can be performed on nanogram quantities of the subject nucleic acids. Paraffin embedded normal tissue or tumor sections can be used, as can fresh or frozen material, tissues, or sections. Because FISH can be applied to limited material, touch preparations prepared from uncultured primary tumors can also be used (*see, e.g., Kallioniemi et al., 1992, Cyto genet. Cell Gener.* 60:190). For instance, small biopsy tissue samples from tumors can be used for touch preparations (*see, e.g., Kallioniemi et al., supra*). Small numbers of cells obtained from aspiration biopsy or cells in bodily fluids (e.g., blood, urine, sputum and the like) can also be analyzed. For prenatal diagnosis, appropriate samples will include amniotic fluid, maternal blood, and the like. Useful hybridization protocols applicable to the methods and reagents disclosed here are described in Pinkel et al., 1988, *Proc. Natl. Acad. Sci. USA*, 85:9138; EPO Pub. No. 430,402; Choo, ed., *METHODS IN MOLECULAR BIOLOGY VOL. 33: IN SITU HYBRIDIZATION PROTOCOLS*, Humana Press, Totowa, New Jersey, (1994); and Kallioniemi et al., *supra*.

Other techniques useful for karyotype analysis include, for example, techniques such as quantitative Southern blotting, quantitative PCR, or comparative genomic hybridization (Kallioniemi et al., 1992, *Science*, 258:818), using the hTERT probes and primers of the invention which may be used to identify amplification, deletion, insertion, substitution or other rearrangement of hTERT sequences in chromosomes in a biological sample.

G. TRT POLYPEPTIDE ASSAYS

1) GENERALLY

The present invention provides methods and reagents for detecting and quantitating hTRT polypeptides. These methods include analytical biochemical methods such as electrophoresis, mass spectroscopy, gel shift, capillary electrophoresis, chromatographic methods such as size exclusion chromatography, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, and the like, or various immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassay (RIA), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, western blotting, mass spectrometry, and others described below and apparent to those of skill in the art upon review of this disclosure.

2) ELECTROPHORETIC ASSAYS

In one embodiment, the hTRT polypeptides are detected in an electrophoretic protein separation; in one aspect, a two-dimensional electrophoresis system is employed. Means of detecting proteins using electrophoretic techniques are well known to those of skill in the art (*see generally*, R. Scopes (1982) *PROTEIN PURIFICATION*, Springer-Verlag, N.Y.; Deutscher, (1990) *METHODS IN ENZYMOLOGY* VOL. 182: *GUIDE TO PROTEIN PURIFICATION*, Academic Press, Inc., N.Y.).

In a related embodiment, a mobility shift assay (*see, e.g.*, Ausubel et al., *supra*) is used. For example, labeled-hTR will associate with hTRT and migrate with altered mobility upon electrophoresis in a nondenaturing polyacrylamide gel or the like. Thus, for example, if an (optionally labeled) hTR probe or a (optionally labeled) telomerase primer is mixed with a sample containing hTRT, or coexpressed with hTRT (*e.g.*, in a cell-free expression system) the presence of hTRT protein (or a polynucleotide encoding hTRT) in the sample will result in a detectable alteration of hTR mobility.

3) IMMUNOASSAYS

a) GENERALLY

The present invention also provides methods for detection of hTRT polypeptides employing one or more antibody reagents of the invention (i.e., immunoassays). As used herein, an immunoassay is an assay that utilizes an antibody (as broadly defined herein and specifically includes fragments, chimeras and other binding agents) that specifically binds an hTRT polypeptide or epitope. Antibodies of the invention may be made by a variety of means well known to those of skill in the art, e.g., as described *supra*.

A number of well established immunological binding assay formats suitable for the practice of the invention are known (see, e.g., U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). See, e.g., METHODS IN CELL BIOLOGY VOLUME 37: ANTIBODIES IN CELL BIOLOGY, Asai, ed. Academic Press, Inc. New York (1993); BASIC AND CLINICAL IMMUNOLOGY 7th Edition, Stites & Terr, eds. (1991); Harlow and Lane, *supra* [e.g., Chapter 14], and Ausubel et al., *supra*, [e.g., Chapter 11], each of which is incorporated by reference in its entirety and for all purposes. Typically, immunological binding assays (or immunoassays) utilize a "capture agent" to specifically bind to and, often, immobilize the analyte. In one embodiment, the capture agent is a moiety that specifically binds to an hTRT polypeptide or subsequence, such as an anti-hTRT antibody. In an alternative embodiment, the capture agent may bind an hTRT-associated protein or RNA under conditions in which the hTRT-associated molecule remains bound to the hTRT (such that if the hTRT-associated molecule is immobilized the hTRT protein is similarly immobilized). It will be understood that in assays in which an hTRT-associated molecule is captured the associated hTRT protein will usually be present and so can be detected, e.g., using an anti-hTRT antibody or the like. Immunoassays for detecting protein complexes are known in the art (see, e.g., Harlow and Lane, *supra*, at page 583).

Usually the hTRT gene product being assayed is detected directly or indirectly using a detectable label. The particular label or detectable group used in the assay is usually not a critical aspect of the invention, so long as it does not significantly interfere with the specific binding of the antibody or antibodies used in the assay. The

label may be covalently attached to the capture agent (e.g., an anti-TRT antibody), or may be attached to a third moiety, such as another antibody, that specifically binds to, e.g., the hTRT polypeptide (at a different epitope than recognized by the capture agent), the capture agent (e.g., an anti-(first antibody) immunoglobulin); an anti-TRT antibody; an antibody that binds an anti-TRT antibody; or, an antibody/telomerase complex (e.g., via binding to an associated molecule such as a telomerase-associated protein). Other proteins capable of binding an antibody used in the assay, such as protein A or protein G, may also be labeled. In some embodiments, it will be useful to use more than one labeled molecule (i.e., ones that can be distinguished from one another). In addition, when the target bound (e.g., immobilized) by the capture agent (e.g., anti-hTRT antibody) is a complex (i.e., a complex of hTRT and a TRT-associated protein, hTR, or other TRT associated molecule), a labeled antibody that recognizes the protein or RNA associated with the hTRT protein can be used. When the complex is a protein-nucleic acid complex (e.g., TRT-hTR), the reporter molecule can be a polynucleotide or other molecule (e.g., enzyme) that recognizes the RNA component of the complex.

Some immunoassay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies. In this case, antigen-coated particles are agglutinated by samples comprising the target antibodies. In this format, the components do not need to be labeled, and the presence of the target antibody can be detected by simple visual inspection.

b) NON-COMPETITIVE ASSAY FORMATS

The present invention provides methods and reagents for competitive and noncompetitive immunoassays for detecting hTRT polypeptides. Noncompetitive immunoassays are assays in which the amount of captured analyte (in this case hTRT) is directly measured. One such assay is a two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on the hTRT protein. See, e.g., Maddox et al., 1983, *J. Exp. Med.*, 158:1211 for background information. In one preferred "sandwich" assay, the capture agent (e.g., an anti-TRT

antibody) is bound directly to a solid substrate where it is immobilized. These immobilized antibodies then capture any hTRT protein present in the test sample. The hTRT thus immobilized can then be labeled, i.e., by binding to a second anti-hTRT antibody bearing a label. Alternatively, the second anti-hTRT antibody may lack a label, but be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second antibody alternatively can be modified with a detectable moiety, such as biotin, to which a third labeled molecule can specifically bind, such as enzyme-labeled streptavidin.

c) COMPETITIVE ASSAY FORMATS

In competitive assays, the amount of hTRT protein present in the sample is measured indirectly by measuring the amount of an added (exogenous) hTRT displaced (or competed away) from a capture agent (e.g., anti-TRT antibody) by the hTRT protein present in the sample. In one competitive assay, a known amount of labeled hTRT protein is added to the sample and the sample is then contacted with a capture agent (e.g., an antibody that specifically binds hTRT protein). The amount of exogenous (labeled) hTRT protein bound to the antibody is inversely proportional to the concentration of hTRT protein present in the sample. In one embodiment, the antibody is immobilized on a solid substrate. The amount of hTRT protein bound to the antibody may be determined either by measuring the amount of hTRT protein present in a TRT/antibody complex, or alternatively by measuring the amount of remaining uncomplexed TRT protein. The amount of hTRT protein may be detected by providing a labeled hTRT molecule.

A hapten inhibition assay is another example of a competitive assay. In this assay hTRT protein is immobilized on a solid substrate. A known amount of anti-TRT antibody is added to the sample, and the sample is then contacted with the immobilized hTRT protein. In this case, the amount of anti-TRT antibody bound to the immobilized hTRT protein is inversely proportional to the amount of hTRT protein present in the sample. The amount of immobilized antibody may be detected by detecting either the immobilized fraction of antibody or the fraction of the antibody that remains in solution. In this aspect, detection may be direct, where the antibody is

labeled, or indirect where the label is bound to a molecule that specifically binds to the antibody as described above.

d) OTHER ASSAY FORMATS

5 The invention also provides reagents and methods for detecting and
quantifying the presence of hTERT in the sample by using an immunoblot (Western blot)
format. In this format, hTERT polypeptides in a sample are separated from other sample
components by gel electrophoresis (e.g., on the basis of molecular weight), the
separated proteins are transferred to a suitable solid support (such as a nitrocellulose
10 filter, a nylon filter, derivatized nylon filter, or the like), and the support is incubated
with anti-TERT antibodies of the invention. The anti-TERT antibodies specifically bind
to hTERT or other TERT on the solid support. These antibodies may be directly labeled
or alternatively may be subsequently detected using labeled antibodies (e.g., labeled
sheep anti-mouse antibodies) or other labeling reagents that specifically bind to the
15 anti-TERT antibody.

Other assay formats include liposome immunoassays (LIA), which use
liposomes designed to bind specific molecules (e.g., antibodies) and release
encapsulated reagents or markers. The released chemicals can then be detected
according to standard techniques (see, Monroe et al., 1986, *Amer. Clin. Prod. Rev.*
20 5:34).

As noted *supra*, assay formats using FACS (and equivalent instruments
or methods) have advantages when measuring hTERT gene products in a heterogeneous
sample (such as a biopsy sample containing both normal and malignant cells).

e) SUBSTRATES, SOLID SUPPORTS, MEMBRANES, FILTERS

25 As noted *supra*, depending upon the assay, various components,
including the antigen, target antibody, or anti-hTERT antibody, may be bound to a solid
surface or support (i.e., a substrate, membrane, or filter paper). Many methods for
immobilizing biomolecules to a variety of solid surfaces are known in the art. For
instance, the solid surface may be a membrane (e.g., nitrocellulose), a microtiter dish
30 (e.g., PVC, polypropylene, or polystyrene), a test tube (glass or plastic), a dipstick (e.g.,

glass, PVC, polypropylene, polystyrene, latex, and the like), a microcentrifuge tube, or a glass or plastic bead. The desired component may be covalently bound or noncovalently attached through nonspecific bonding.

5 A wide variety of organic and inorganic polymers, both natural and synthetic may be employed as the material for the solid surface. Illustrative polymers include polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), rayon, nylon, poly(vinyl butyrate), polyvinylidene difluoride (PVDF), silicones, polyformaldehyde, cellulose, cellulose acetate, nitrocellulose, and the like. Other materials which may be employed, include
10 paper, glasses, ceramics, metals, metalloids, semiconductive materials, cements or the like. In addition, substances that form gels, such as proteins (e.g., gelatins), lipopolysaccharides, silicates, agarose and polyacrylamides can be used. Polymers which form several aqueous phases, such as dextrans, polyalkylene glycols or surfactants, such as phospholipids, long chain (12-24 carbon atoms) alkyl ammonium
15 salts and the like are also suitable. Where the solid surface is porous, various pore sizes may be employed depending upon the nature of the system.

In preparing the surface, a plurality of different materials may be employed, particularly as laminates, to obtain various properties. For example, protein coatings, such as gelatin can be used to avoid non-specific binding, simplify covalent
20 conjugation, enhance signal detection or the like.

If covalent bonding between a compound and the surface is desired, the surface will usually be polyfunctional or be capable of being polyfunctionalized. Functional groups which may be present on the surface and used for linking can include
25 carboxylic acids, aldehydes, amino groups, cyano groups, ethylenic groups, hydroxyl groups, mercapto groups and the like. The manner of linking a wide variety of compounds to various surfaces is well known and is amply illustrated in the literature. See, for example, *Immobilized Enzymes*, Ichiro Chibata, Halsted Press, New York, 1978, and Cuatrecasas (1970) *J. Biol. Chem.* 245 3059).

In addition to covalent bonding, various methods for noncovalently
30 binding an assay component can be used. Noncovalent binding is typically nonspecific absorption of a compound to the surface.

One of skill in the art will appreciate that it is often desirable to reduce non-specific binding in immunoassays. Particularly, where the assay involves an antigen or antibody immobilized on a solid substrate it is desirable to minimize the amount of non-specific binding to the substrate. Means of reducing such non-specific binding are well known to those of skill in the art. Typically, this involves coating the substrate with a proteinaceous composition. In particular, protein compositions such as bovine serum albumin (BSA), nonfat powdered milk, and gelatin are widely used with powdered milk sometimes preferred. Alternatively, the surface is designed such that it nonspecifically binds one component but does not significantly bind another. For example, a surface bearing a lectin such as Concanavalin A will bind a carbohydrate containing compound but not a labeled protein that lacks glycosylation. Various solid surfaces for use in noncovalent attachment of assay components are reviewed in U.S. Patent Nos. 4,447,576 and 4,254,082.

H) ASSAYS FOR ANTI-TRT ANTIBODIES

The present invention also provides reagents and assays for detecting hTERT-specific immunoglobulins. In one embodiment, immobilized hTERT (e.g., recombinant hTERT bound to a microassay plate well) is incubated with serum from a patient under conditions in which anti-hTERT antibodies, if present, bind the immobilized hTERT. After washing to remove nonspecifically bound immunoglobulin, bound serum antibodies can be detected, if they are present, by adding detectably labeled anti-(human Ig) antibodies (alternative embodiments and variations are well known to those of skill in the art; see, e.g., Harlow, *supra*, at Ch. 14). These assays are useful for detecting anti-hTERT antibodies in any source including animal or human serum or a carrier such as saline. In one embodiment, the assays are used to detect or monitor an immune response to hTERT proteins in a patient, particularly an autoimmune (e.g., anti-telomerase) response. Anti-hTERT antibodies may be present in the serum or other tissues or fluids from a patient suffering from an autoimmune disease or other condition.

I) ASSAY COMBINATIONS

The diagnostic and prognostic assays described herein can be carried out in various combinations and can also be carried out in conjunction with other diagnostic or prognostic tests. For example, when the present methods are used to detect the presence of cancer cells in patient sample, the presence of hTERT can be used to determine the stage of the disease, whether a particular tumor is likely to invade adjoining tissue or metastasize to a distant location, and whether a recurrence of the cancer is likely. Tests that may provide additional information include microscopic analysis of biopsy samples, detection of antigens (e.g., cell-surface markers) associated with tumorigenicity (e.g., using histocytochemistry, FACS, or the like), imaging methods (e.g., upon administration to a patient of labeled anti-tumor antibodies), telomerase activity assays, telomere length assays, hTR assays, or the like. Such combination tests can provide useful information regarding the progression of a disease.

It will also be recognized that combinations of assays can provide useful information. For example, and as noted above, assays for hTERT mRNA can be combined with assays for hTR (human telomerase RNA) or telomerase activity (i.e., TRAP) assays to provide information about telomerase assembly and function.

J) KITS

The present invention also provides kits useful for the screening, monitoring, diagnosis and prognosis of patients with a telomerase-related condition, or for determination of the level of expression of hTERT in cells or cell lines. The kits include one or more reagents for determining the presence or absence of an hTERT gene product (RNA or protein) or for quantifying expression of the hTERT gene. Preferred reagents include nucleic acid primers and probes that specifically bind to the hTERT gene, RNA, cDNA, or portions thereof, along with proteins, peptides, antibodies, and control primers, probes, oligonucleotides, proteins, peptides and antibodies. Other materials, including enzymes (e.g., reverse transcriptases, DNA polymerases, ligases), buffers, reagents (labels, dNTPs), may be included.

The kits may include alternatively, or in combination with any of the

other components described herein, an antibody that specifically binds to hTERT polypeptides or subsequences thereof. The antibody can be monoclonal or polyclonal. The antibody can be conjugated to another moiety such as a label and/or it can be immobilized on a solid support (substrate). The kit(s) may also contain a second antibody for detection of hTERT polypeptide/antibody complexes or for detection of hybridized nucleic acid probes, as well as one or more hTERT peptides or proteins for use as control or other reagents.

The antibody or hybridization probe may be free or immobilized on a solid support such as a test tube, a microtiter plate, a dipstick and the like. The kit may also contain instructional materials teaching the use of the antibody or hybridization probe in an assay for the detection of TERT. The kit may contain appropriate reagents for detection of labels, or for labeling positive and negative controls, washing solutions, dilution buffers and the like.

In one embodiment, the kit includes a primer pair for amplifying hTERT mRNA. Such a kit may also include a probe for hTERT amplified DNA and/or a polymerase, buffer, dNTPs, and the like. In another, the kit comprises a probe, optionally a labeled probe. In another, the kit comprises an antibody.

X. IDENTIFICATION OF MODULATORS OF TELOMERASE ACTIVITY

A. GENERALLY

The invention provides compounds and treatments that modulate the activity or expression of a telomerase or telomerase component (e.g., hTERT protein). The invention also provides assays and screening methods (including high-throughput screens) for identification of compounds and treatments that modulate telomerase activity or expression. These modulators of telomerase activity and expression (hereinafter referred to as "modulators") include telomerase agonists (which increase telomerase activity and/or expression) and telomerase antagonists (which decrease telomerase activity and/or expression).

The modulators of the invention have a wide variety of uses. For example, it is contemplated that telomerase modulators will be effective therapeutic agents for treatment of human diseases. Screening for agonist activity and

transcriptional or translational activators provides for compositions that increase telomerase activity in a cell (including a telomere dependent replicative capacity, or a "partial" telomerase activity). Such agonist compositions provide for methods of immortalizing otherwise normal untransformed cells, including cells which can
5 express useful proteins. Such agonists can also provide for methods of controlling cellular senescence. Conversely, screening for antagonist activity provides for compositions that decrease telomere dependent replicative capacity, thereby mortalizing otherwise immortal cells, such as cancer cells. Screening for antagonist activity provides for compositions that decrease telomerase activity, thereby preventing
10 unlimited cell division of cells exhibiting unregulated cell growth, such as cancer cells. Illustrative diseases and conditions that may be treated using modulators are listed herein. e.g., in Sections VII and IX, *supra*. In general, the modulators of the invention can be used whenever it is desired to increase or decrease a telomerase activity in a cell or organism. Thus, in addition to use in treatment of disease, a modulator that increases
15 hTERT expression levels can be used to produce a cultured human cell line having properties as generally described in Section VIII, *supra*, and various other uses that will be apparent to one of skill.

A compound or treatment modulates "expression" of telomerase or a telomerase component when administration of the compound or treatment changes the
20 rate or level of transcription of the gene encoding a telomerase component (e.g., the gene encoding hTERT mRNA), affects stability or post-transcriptional processing of RNA encoding a telomerase component (e.g., transport, splicing, polyadenylation, or other modification), affects translation, stability, post-translational processing or modification of an encoded protein (e.g., hTERT), or otherwise changes the level of
25 functional (e.g., catalytically active) telomerase RNP. A compound or treatment affects a telomerase "activity" when administration of the compound or treatment changes a telomerase activity such as any activity described in Section IV(B), *supra* (e.g., including processive or non-processive telomerase catalytic activity; telomerase processivity; conventional reverse transcriptase activity; nucleolytic activity; primer or
30 substrate binding activity; dNTP binding activity; RNA binding activity; telomerase RNP assembly; and protein binding activity). It will be appreciated that there is not

—
—
— necessarily a sharp delineation between changes in "activity" and changes in
— "expression," and that these terms are used for ease of discussion and not for limitation.
— It will also be appreciated that the modulators of the invention should specifically affect
— telomerase activity or expression (e.g., without generally changing the expression of
5 housekeeping proteins such as actin) rather than, for example, reducing expression of a
— telomerase component by nonspecific poisoning of a target cell.

—
— **B. ASSAYS FOR IDENTIFICATION OF TELOMERASE**
— **MODULATORS**

— 10 The invention provides methods and reagents to screen for compositions
— or compounds capable of affecting expression of a telomerase or telomerase
— component, capable of modifying the DNA replicative capacity of telomerase, or
— otherwise modifying the ability of the telomerase enzyme and TRT protein to
— synthesize telomeric DNA ("full activity"). The invention also provides screens for
15 modulators of any or all of hTRT's "partial activities." Thus, the present invention
— provides assays that can be used to screen for agents that increase the activity of
— telomerase, for example, by causing hTRT protein or telomerase to be expressed in a
— cell in which it normally is not expressed or by increasing telomerase activity levels in
— telomerase positive cells.

— 20 Telomerase or telomerase subunit proteins or their catalytic or
— immunogenic fragments or oligopeptides thereof, can be used for screening therapeutic
— compounds in any of a variety of drug screening techniques. The fragment employed
— in such a test may be free in solution, affixed to a solid support, borne on a cell surface,
— or located intracellularly. The formation of binding complexes, between telomerase or
25 the subunit protein and the agent being tested, may be measured.

— In various embodiments, the invention includes methods for screening
— for antagonists that: bind to the enzyme's active site; inhibit the association of its RNA
— moiety, telomerase-associated proteins, nucleotides, or telomeric DNA to telomerase or
— hTRT protein; promote the disassociation of the enzyme complex; interfere with
30 transcription of the telomerase RNA moiety (e.g., hTR); or inhibit any of the "partial
— activities" described herein. The invention provides methods for screening for

compositions that inhibit the association of nucleic acid and/or telomerase-associated compositions with hTERT, such as the association of hTR with hTERT or the association of hTERT with the human homologs of p80 or p95 or another associated protein, or association of hTERT with a telomere or a nucleotide; screening for compositions that promote the disassociation or promote the association (i.e., assembly) of the enzyme complex, such as an antibody directed to hTR or hTERT; screening for agents that effect the processivity of the enzyme; and screening for nucleic acids and other compositions that bind to telomerase, such as a nucleic acid complementary to hTR. The invention further contemplates screening for compositions that increase or decrease the transcription of the hTERT gene and/or translation of the hTERT gene product. The invention also contemplates a method of screening for telomerase modulators in animals, in one embodiment, by reconstituting a telomerase activity, or an anti-telomerase activity, in an animal, such as a transgenic animal. The invention provides for *in vivo* assays systems that include "knockout" models, in which one or several units of the endogenous telomerase, telomerase RNA moiety and/or telomerase-associated proteins have been deleted or inhibited. The endogenous telomerase activity, full or partial, can remain or be absent. In one embodiment, an exogenous telomerase activity, full or partial, is reconstituted.

In one embodiment of the invention, a variety of partial activity telomerase assays are provided to identify a variety of different classes of modulators of telomerase activity. The "partial activity" assays of the invention allow identification of classes of telomerase activity modulators that might otherwise not be detected in a "full activity" telomerase assay. One partial activity assay involves the non-processive activity of TRT and telomerase. The processive nature of telomerase is described by Morin (1989) *Cell* 59:521-529; see also Prowse (1993) "Identification of a nonprocessive telomerase activity from mouse cells" *Proc. Natl. Acad. Sci. USA* 90:1493-1497. Another partial activity assay of the invention exploits the "reverse-transcriptase-like" activity of telomerase. In these assays, one assays the reverse transcriptase activity of the hTERT protein. See Lingner (1997) "Reverse transcriptase motifs in the catalytic subunit of telomerase" *Science* 276:561-567. Another partial activity assay of the invention exploits the "nucleolytic activity" of hTERT and

telomerase, involving the enzyme's removing of at least one nucleotide, typically guanosine, from the 3' strand of a primer. This nucleolytic activity has been observed in *Tetrahymena* telomerase by Collins (1993) "*Tetrahymena* telomerase catalyzes nucleolytic cleavage and nonprocessive elongation" *Genes Dev* 7:1364-1376. Another partial activity assay of the invention involves analyzing hTRT's and telomerase's ability to bind nucleotides as part of its enzymatic processive DNA polymerization activity. Another partial activity assay of the invention involves analyzing hTRT's or telomerase's ability to bind its RNA moiety, *i.e.*, hTR for human cells, used as a template for telomere synthesis. Additional partial activity assays of the invention involve analyzing hTRT's and telomerase's ability to bind chromosomes *in vivo*, or to bind oligonucleotide primers *in vitro* or in reconstituted systems, or to bind proteins associated with chromosomal structure (see, for an example of such a protein, Harrington (1995) *J Biol Chem* 270: 8893-8901). Chromosomal structures which bind hTRT include, for example, telomeric repeat DNA, telomere proteins, histones, nuclear matrix protein, cell division/ cell cycle control proteins and the like.

In one embodiment, an assay for identification of modulators comprises contacting one or more cells (*i.e.*, "test cells") with a test compound, and determining whether the test compound affects expression or activity of a telomerase (or telomerase component) in the cell. Usually this determination comprises comparing the activity or expression in the test cell compared to a similar cell or cells (*i.e.*, control cells) that have not been contacted with the test compound. Alternatively, cell extracts may be used in place of intact cells. In a related embodiment, the test compound is administered to a multicellular organism (*e.g.*, a plant or animal). The telomerase or telomerase component may be wholly endogenous to the cell or multicellular organism (*i.e.*, encoded by naturally occurring endogenous genes), or may be a recombinant cell or transgenic organism comprising one or more recombinantly expressed telomerase components (*e.g.*, hTRT, hTR, telomerase-associated proteins), or may have both endogenous and recombinant components. Thus, in one embodiment, telomerase-activity-modulators are administered to mortal cells. In another embodiment, telomerase-activity-modulators are administered to immortal cells. For example, antagonists of telomerase-mediated DNA replication can be identified by administering

the putative inhibitory composition to a cell that is known to exhibit significant amounts of telomerase activity, such as cancer cells, and measuring whether a decrease in telomerase activity, telomere length, or proliferative capacity is observed, all of which are indicative of a compound with antagonist activity.

5 In another embodiment, a modulator is identified by monitoring a change in a telomerase activity of a ribonucleoprotein complex (RNP) comprising a TRT (e.g., hTRT) and a template RNA (e.g., hTR), which RNP is reconstituted *in vitro* (e.g., as described in Example 7, *infra*).

10 In yet another embodiment, the modulator is identified by monitoring a change in expression of a TRT gene product (e.g., RNA or protein) in a cell, animal, *in vitro* expression system, or other expression system.

15 In still another embodiment, the modulator is identified by changing the expression of a reporter gene, such as that described in Example 15, whose expression is regulated, in whole or part, by a naturally occurring TRT regulatory element such as a promoter or enhancer. In a related embodiment, the ability of a test compound to bind to a telomerase component (e.g., hTRT), RNA, or gene regulatory sequence (e.g., the TRT gene promoter) is assayed.

20 In another embodiment, the modulator is identified by observing changes in hTRT pre-mRNA processing, for example, alternatively spliced products, alternative poly-adenylation events, RNA cleavage, and the like. In a related embodiment the activity of the modulator can be observed by monitoring the production of variant hTRT polypeptides, some of which may possess dominant-negative telomerase regulation activity.

25 Assay formats for identification of compounds that affect expression and activity of proteins are well known in the biotechnological and pharmaceutical industries, and numerous additional assays and variations of the illustrative assays provided *supra* will be apparent to those of skill.

30 Changes in telomerase activity or expression can be measured by any suitable method. Changes in levels of expression of a telomerase component (e.g., hTRT protein) or precursor (e.g., hTRT mRNA) can be assayed using methods well known to those of skill, some of which are described hereinabove, e.g., in Section IX

and including monitoring levels of TRT gene products (e.g., protein and RNAs) by hybridization (e.g., using the TRT probes and primers of the invention), immunoassays (e.g., using the anti-TRT antibodies of the invention), RNase protection assays, amplification assays, or any other suitable detection means described herein or known in the art. Quantitating amounts of nucleic acid in a sample (e.g., evaluating levels of RNA, e.g., hTR or hTRT mRNA) is also useful in evaluating *cis*- or *trans*-transcriptional regulators.

Similarly, changes in telomerase activity can be measured using methods such as those described herein (e.g., in Section IV(B), *supra*) or other assays of telomerase function. Quantitation of telomerase activity, when desired, may be carried out by any method, including those disclosed herein. Telomerase antagonists that can cause or accelerate loss of telomeric structure can be identified by monitoring and measuring their effect on telomerase activity *in vivo*, *ex vivo*, or *in vitro*, or by their effects on telomere length (as measured or detected through staining, use of tagged hybridization probes or other means) or, simply, by the inhibition of cell division of telomerase positive cancer cells (critical shortening of telomeres leads to a phenomenon termed "crisis" or M2 senescence (Shay, 1991) *Biochem Biophys Acta* 1072:1-7), which cancer cells have bypassed by the activation of telomerase, but which, in the absence of telomerase, will lead to their senescence or death through chromosomal deletion and rearrangement). The *in vivo* human telomerase activity reconstitution provides for a method of screening for telomerase modulators in cells or animals from any origin. Such agonists can be identified in an activity assay of the invention, including measurements of changes in telomere length. Other examples of assays measuring telomerase activity in cells include assays for the accumulation or loss of telomere structure, the TRAP assay or a quantitative polymerase chain reaction assay.

In one embodiment, the assays of the invention also include a method where the test compound produces a statistically significant decrease in the activity of hTRT as measured by the incorporation of a labeled nucleotide into a substrate compared to the relative amount of incorporated label in a parallel reaction lacking the test compound, thereby determining that the test compound is a telomerase inhibitor

The methods of the invention are amenable to adaptations from

protocols described in the scientific and patent literature and known in the art. For example, when a telomerase or TRT protein of this invention is used to identify compositions which act as modulators of telomerase activities, large numbers of potentially useful molecules can be screened in a single test. The modulators can have an inhibitory (antagonist) or potentiating (agonist) effect on telomerase activity. For example, if a panel of 1,000 inhibitors is to be screened, all 1,000 inhibitors can potentially be placed into one microtiter well and tested simultaneously. If such an inhibitor is discovered, then the pool of 1,000 can be subdivided into 10 pools of 100 and the process repeated until an individual inhibitor is identified.

In drug screening large numbers of compounds are examined for their ability to act as telomerase modulators, a process greatly accelerated by the techniques of high throughput screening. The assays for telomerase activity, full or partial, described herein may be adapted to be used in a high throughput technique. Those skilled in the art appreciate that there are numerous methods for accomplishing this purpose.

Another technique for drug screening which may be applied for high throughput screening of compounds having suitable binding affinity to the telomerase or telomerase protein subunit is described in detail in "Determination of Amino Acid Sequence Antigenicity" by Geysen, (Geysen, WO Application 84/03564, published on September 13, 1984, incorporated herein by reference). In summary, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with fragments of telomerase or telomerase protein subunits and washed. Bound telomerase or telomerase protein subunit is then detected by methods well known in the art. Substantially purified telomerase or telomerase protein subunit can also be coated directly onto plates for use in the aforementioned drug screening techniques. Alternatively, non-neutralizing antibodies can be used to capture the peptide and immobilize it on a solid support.

This invention also contemplates the use of competitive drug screening assays in which neutralizing antibodies capable of binding telomerase or subunit protein(s) specifically compete with a test compound for binding telomerase or the

subunit protein. Antibodies can also be used to detect the presence of any peptide which shares one or more antigenic determinants with the telomerase or subunit protein.

Additional methods for identifying modulators of a telomerase activity have been described in U.S. Patent No. 5,645,986, which is incorporated herein by reference. It will be appreciated that the present invention provides improvements to previously known methods, in part by providing reagents such as hTRT polynucleotides, probes and primers, highly purified hTR, hTRT and telomerase, as well as anti-telomerase and anti-TRT antibodies, all of which may be used in assays, e.g., as controls, standards, binding or hybridization agents, or otherwise.

It will be recognized that the recombinantly produced telomerase and TRT (e.g., hTRT) of the invention will be useful in assays for identification of modulators. The screening assay can utilize telomerase or hTRT derived by a full or partial reconstitution of telomerase activity, or by an augmentation of existing activity. The assay or screens provided by the invention can be used to test for the ability of telomerase to synthesize telomeric DNA or to test for any one or all or of the "partial activities" of hTRT and TRTs generally, as described above. The assay can incorporate *ex vivo* modification of cells which have been manipulated to express telomerase with or without its RNA moiety or associated proteins, and these can be re-implanted into an animal, which can be used for *in vivo* testing. Thus, this invention provides *in vivo* assays and transgenic animals useful therein. These *in vivo* assays systems can employ "knockout" cells, in which one or several units of the endogenous telomerase enzyme complex have been deleted or inhibited, as well as cells in which an exogenous or endogenous telomerase activity is reconstituted or activated.

Telomerases and TRT proteins that have been modified in a site-specific manner (by site-specific mutation) to modify or delete any or all functions of the telomerase enzyme or the TRT protein can also be employed in the screens of the invention to discover therapeutic agents. For example, the TRT can be engineered to lose its ability to bind substrate DNA, to bind its RNA moiety (as hTR), to catalyze the addition of telomeric DNA, to bind deoxynucleotide substrate, to have nucleolytic activity, to bind telomere-associated proteins or chromosomal structures, and the like.

The resulting "mutant proteins" or "muteens" can be used to identify compounds that specifically modulate one, several, or all functions or activities of the TRT protein or telomerase.

C. EXEMPLARY TELOMERASE MODULATORS

1) GENERALLY

The test compounds referred to *supra* may be any of a large variety of compounds, both naturally occurring and synthetic, organic and inorganic, and including polymers (e.g., oligopeptides, polypeptides, oligonucleotides, and polynucleotides), small molecules, antibodies (as broadly defined herein), sugars, fatty acids, nucleotides and nucleotide analogs, analogs of naturally occurring structures (e.g., peptide mimetics, nucleic acid analogs, and the like), and numerous other compounds.

The invention provides modulators of all types, without limitation to any particular mechanism of action. For illustrative purposes, examples of modulators include compounds or treatments that:

(i) bind to the hTRT polypeptide (e.g., the active site of the enzyme) or other telomerase component, and affect a telomerase activity;

(ii) inhibit or promote association, or inhibit or promote disassociation, of a telomerase component (e.g., hTRT or the hTRT-hTR RNP) with or from a telomerase-associated protein (e.g., including those described in Section IV(D), *supra*);

(iii) inhibit or promote association, or inhibit or promote disassociation, of telomerase polypeptides (e.g., hTRT) with or from a telomerase RNA (e.g., hTR);

(iv) inhibit or promote association, or inhibit or promote disassociation, of telomerase polypeptides (e.g., hTRT) with or from chromosomes (e.g., telomeres) or chromosomal DNA (e.g., telomeric DNA);

(v) increase or decrease expression of a telomerase component gene product (e.g., products of the hTRT gene), including change the rate or level of transcription of the TRT gene, or translation, transport or stability of a gene

product, or the like, by binding to the gene or gene product (e.g., by interacting with a factor (e.g., a transcription regulatory protein) that affects transcription of the hTERT gene or another telomerase component).

2) PEPTIDE MODULATORS

5 Potential modulators of telomerase activity also include peptides (e.g., inhibitory (antagonist) and activator (agonist) peptide modulators). For example, oligopeptides with randomly generated sequences can be screened to discover peptide modulators (agonists or inhibitors) of telomerase activity. Such peptides can be used directly as drugs or to find the orientation or position of a functional group that can
10 inhibit telomerase activity that, in turn, leads to design and testing of a small molecule inhibitor, or becomes the backbone for chemical modifications that increase pharmacological utility. Peptides can be structural mimetics, and one can use molecular modeling programs to design mimetics based on the characteristic secondary structure and/or tertiary structure of telomerase enzyme and hTERT protein. Such
15 structural mimetics can also be used therapeutically, *in vivo*, as modulators of telomerase activity (agonists and antagonists). Structural mimetics can also be used as immunogens to elicit anti-telomerase or anti-TERT protein antibodies

3) INHIBITORY NATURAL COMPOUNDS AS MODULATORS OF TELOMERASE ACTIVITY

20 In addition, a large number of potentially useful activity-modifying compounds can be screened in extracts from natural products as a source material. Sources of such extracts can be from a large number of species of fungi, actinomycetes, algae, insects, protozoa, plants, and bacteria. Those extracts showing inhibitory activity can then be analyzed to isolate the active molecule. See for example, Turner (1996) *J. Ethnopharmacol* 51(1-3):39-43; Suh (1995) *Anticancer Res.* 15:233-239.
25

4) INHIBITORY OLIGONUCLEOTIDES

One particularly useful set of inhibitors provided by the present invention includes oligonucleotides which are able to either bind mRNA encoding hTERT protein or to the hTERT gene, in either case preventing or inhibiting the
30 production of functional hTERT protein. Other oligonucleotides of the invention interact with telomerase's RNA moiety, such as hTR, or are able to prevent binding of

telomerase or hTERT to its DNA target, or one telomerase component to another, or to a substrate. Such oligonucleotides can also bind the telomerase enzyme, hTERT protein, or both protein and RNA and inhibit a partial activity as described above (such as its processive activity, its reverse transcriptase activity, its nucleolytic activity, and the like). The association can be through sequence specific hybridization to another nucleic acid or by general binding, as in an aptamer, or both.

Telomerase activity can be inhibited by targeting the hTERT mRNA with antisense oligonucleotides capable of binding the hTERT mRNA.

Another useful class of inhibitors includes oligonucleotides which cause inactivation or cleavage of hTERT mRNA or hTR. That is, the oligonucleotide is chemically modified, or has enzyme activity, which causes such cleavage, such as is the case for a ribozyme, an EDTA-tethered oligonucleotide, or a covalently bound oligonucleotide, such as a psoralen or other cross-linking reagent bound oligonucleotide. As noted above, one may screen a pool of many different such oligonucleotides for those with the desired activity.

Another useful class of inhibitors includes oligonucleotides which bind polypeptides. Double- or single-stranded DNA or double- or single-stranded RNA molecules that bind to specific polypeptides targets are called "aptamers." The specific oligonucleotide-polypeptide association may be mediated by electrostatic interactions. For example, aptamers specifically bind to anion-binding exosites on thrombin, which physiologically binds to the polyanionic heparin (Bock (1992) *Nature* 355:564-566). Because hTERT protein binds both hTR and its DNA substrate, and because the present invention provides hTERT and other TRT proteins in purified form in large quantities, those of skill in the art can readily screen for TRT-binding aptamers using the methods of the invention.

Oligonucleotides (e.g., RNA oligonucleotides) that bind telomerase, hTERT, hTR, or portions thereof, can be generated using the techniques of SELEX (Tuerk, 1997, *Methods Mol Biol* 67, 2190). In this technique a very large pool (10⁶-10⁹) of random sequence nucleic acids is bound to the target (e.g. hTERT) using conditions that cause a large amount of discrimination between molecules with high affinity and low affinity for binding the target. The bound molecules are separated

from unbound, and the bound molecules are amplified by virtue of a specific nucleic acid sequence included at their termini and suitable amplification reagents. This process is reiterated several times until a relatively small number of molecules remain that possess high binding affinity for the target. These molecules can then be tested for their ability to modulate telomerase activity as described herein.

Antagonists of telomerase-mediated DNA replication can also be based on inhibition of hTR (Norton (1996) *Nature Biotechnology* 14:615-619) through complementary sequence recognition or cleavage, as through ribozymes.

The inhibitory oligonucleotides of the invention can be transferred into the cell using a variety of techniques well known in the art. For example, oligonucleotides can be delivered into the cytoplasm without specific modification. Alternatively, they can be delivered by the use of liposomes which fuse with the cellular membrane or are endocytosed, *i.e.*, by employing ligands attached to the liposome or directly to the oligonucleotide, that bind to surface membrane protein receptors of the cell resulting in endocytosis. Alternatively, the cells may be permeabilized to enhance transport of the oligonucleotides into the cell, without injuring the host cells. One can use a DNA binding protein, *e.g.*, HBCGF-1, known to transport an oligonucleotide into a cell.

5) INHIBITORY RIBOZYMES

Ribozymes act by binding to a target RNA through the target RNA binding portion of a ribozyme which is held in close proximity to an enzymatic portion of the ribozyme that cleaves the target RNA. Thus, the ribozyme recognizes and binds a target RNA usually through complementary base-pairing, and once bound to the correct site, acts enzymatically to cleave and inactivate the target RNA. Cleavage of a target RNA in such a manner will destroy its ability to direct synthesis of an encoded protein if the cleavage occurs in the coding sequence. After a ribozyme has bound and cleaved its RNA target, it is typically released from that RNA and so can bind and cleave new targets repeatedly.

6) IDENTIFYING TELOMERASE-ASSOCIATED PROTEINS FOR USE AS MODULATORS

In one embodiment of the invention, telomerase is used to identify telomerase-associated proteins, *i.e.*, telomerase accessory proteins which modulate or otherwise complement telomerase activity. As noted above, these proteins or fragments thereof can modulate function by causing the dissociation or preventing the association of the telomerase enzyme complex, preventing the assembly of the telomerase complex, preventing hTERT from binding to its nucleic acid complement or to its DNA template, preventing hTERT from binding nucleotides, or preventing, augmenting, or inhibiting any one, several or all of the partial activities of the telomerase enzyme or hTERT protein, as described above.

One of skill in the art can use the methods of the invention to identify which portions (*e.g.*, domains) of these telomerase-associating proteins contact telomerase. In one embodiment of the invention, these telomerase-associating proteins or fragments thereof are used as modulators of telomerase activity.

7) TELOMERASE-ASSOCIATED PROTEINS AS DOMINANT NEGATIVE MUTANTS

In one embodiment of the invention, telomerase-associated proteins are used as modulators of telomerase activity. Telomerase-associated proteins include chromosomal structures, such as histones, nuclear matrix proteins, cell division and cell cycle control proteins, and the like. Other telomerase-associated proteins which can be used as modulators for the purpose of the invention include the p80 and p95 proteins and their human homologs, such as TP1 and TRF-1 (Chong, 1995, *Science* 270:1663-1667). In addition, fragments of these telomerase-associated proteins can be identified by the skilled artisan in accordance with the methods of the invention and used as modulators of telomerase activity.

8) DOMINANT NEGATIVE MUTANTS

Eight highly conserved motifs have been identified between TRTs of different non-human species, as described above (see also Lingner (1997) *Science* 276:561-567). Figure 4 shows a schematic of the human TRT amino acid sequence (from pGRN121) and RT motifs as compared to *S. pombe* Trt1p, *Euplotes* p123 and *S.*

cerevisiae Est2 p. The present invention provides recombinant and synthetic nucleic acids in which the codons for the conserved amino acid residues in each, alone or in conjunction with one or more additional codons, of all eight of these motifs has been a changed to each of the other codons. A variety of the resulting coding sequences
5 express a non-functional hTRT. See, for instance, Example 16. Thus, the present invention provides, for example, a wide variety of "mutated" telomerase enzymes and TRT proteins which have a partial activity but not full activity of telomerase. For example, one such telomerase is able to bind telomeric structures, but not bind telomerase-associated RNA (*i.e.*, hTR). If expressed at high enough levels, such a
10 telomerase mutant can deplete a necessary telomerase component (*e.g.*, hTR) and thereby function as an inhibitor of wild-type telomerase activity. A mutated telomerase acting in this manner is an antagonist or a so-called "dominant-negative" mutant.

9) ANTIBODIES

In general, the antibodies of the invention can be used to identify, purify,
15 or inhibit any or all activity of telomerase enzyme and hTRT protein. Antibodies can act as antagonists of telomerase activity in a variety of ways, for example, by preventing the telomerase complex or nucleotide from binding to its DNA substrates, by preventing the components of telomerase from forming an active complex, by maintaining a functional (telomerase complex) quaternary structure or by binding to
20 one of the enzyme's active sites or other sites that have allosteric effects on activity (the different partial activities of telomerase are described in detail elsewhere in this specification).

D) MODULATOR SYNTHESIS

25 It is contemplated that the telomerase modulators of the invention will be made using methods well known in the pharmaceutical arts, including combinatorial methods and rational drug design techniques.

1) COMBINATORIAL CHEMISTRY METHODOLOGY

The creation and simultaneous screening of large libraries of synthetic
30 molecules can be carried out using well-known techniques in combinatorial chemistry, for example, see van Breemen (1997) *Anal Chem* 69:2159-2164; Lam (1997)

Anticancer Drug Des 12:145-167 (1997).

As noted above, combinatorial chemistry methodology can be used to create vast numbers of oligonucleotides (or other compounds) that can be rapidly screened for specific oligonucleotides (or compounds) that have appropriate binding
5 affinities and specificities toward any target, such as the TRT proteins of the invention, can be utilized (for general background information Gold (1995) *J. of Biol. Chem.* 270:13581-13584).

2) RATIONAL DRUG DESIGN

Rational drug design involves an integrated set of methodologies that
10 include structural analysis of target molecules, synthetic chemistries, and advanced computational tools. When used to design modulators, such as antagonists/inhibitors of protein targets, such as telomerase enzyme and hTRT protein, the objective of rational drug design is to understand a molecule's three-dimensional shape and chemistry. Rational drug design is aided by X-ray crystallographic data or NMR data, which can
15 now be determined for the hTRT protein and telomerase enzyme in accordance with the methods and using the reagents provided by the invention. Calculations on electrostatics, hydrophobicities and solvent accessibility is also helpful. See, for example, Coldren (1997) *Proc. Natl. Acad. Sci. USA* 94:6635-6640.

20 E) KITS

The invention also provides kits that can be used to aid in determining whether a test compound is a modulator of a TRT activity. The kit will typically include one or more of the following components: a substantially purified TRT polypeptide or polynucleotide (including probes and primers); a plasmid capable of
25 expressing a TRT (e.g., hTRT) when introduced into a cell or cell-free expression system; a plasmid capable of expressing a TR (e.g., hTR) when introduced into a cell or cell-free expression system; cells or cell lines; a composition to detect a change in TRT activity; and, an instructional material teaching a means to detect and measure a change in the TRT activity, indicating that a change in the telomerase activity in the presence
30 of the test compound is an indicator that the test compound modulates the telomerase activity, and one or more containers. The kit can also include means, such as TRAP

assay reagents or reagents for a quantitative polymerase chain reaction assay, to measure a change in TRT activity. The kit may also include instructional material teaching a means to detect and measure a change in the TRT activity, indicating that a change in the telomerase activity in the presence of the test compound is an indicator that the test compound modulates the telomerase activity.

XI. TRANSGENIC ORGANISMS (TELOMERASE KNOCKOUT CELLS AND ANIMAL MODELS)

The invention also provides transgenic non-human multicellular organisms (e.g., plants and non-human animals) or unicellular organisms (e.g., yeast) comprising an exogenous TRT gene sequence, which may be a coding sequence or a regulatory (e.g., promoter) sequence. In one embodiment, the organism expresses an exogenous TRT polypeptide, having a sequence of a human TRT protein. In a related embodiment, the organism also expresses a telomerase RNA component (e.g., hTR).

The invention also provides unicellular and multicellular organisms (or cells therefrom) in which at least one gene encoding a telomerase component (e.g., TRT or TR) or telomerase-associated protein is mutated or deleted (i.e., in a coding or regulatory region) such that native telomerase is not expressed, or is expressed at reduced levels or with different activities when compared to wild-type cells or organisms. Such cells and organisms are often referred to as "gene knock-out" cells or organisms.

The invention further provides cells and organisms in which an endogenous telomerase gene (e.g., murine TRT) is either present or optionally mutated or deleted and an exogenous telomerase gene or variant (e.g., human TRT) is introduced and expressed. Cells and organisms of this type will be useful, for example, as model systems for identifying modulators of hTRT activity or expression; determining the effects of mutations in telomerase component genes, and other uses such as determining the developmental timing and tissue location of telomerase activity (e.g., for assessing when to administer a telomerase modulator and for assessing any potential side effects).

Examples of multicellular organisms include plants, insects, and

nonhuman animals such as mice, rats, rabbits, monkeys, apes, pigs, and other nonhuman mammals. An example of a unicellular organism is a yeast.

Methods for alteration or disruption of specific genes (e.g., endogenous TRT genes) are well known to those of skill, see, e.g., Baudin et al., 1993, *Nucl. Acids Res.* 21:3329; Wach et al., 1994, *Yeast* 10:1793; Rothstein, 1991, *Methods Enzymol.* 194:281; Anderson, 1995, *Methods Cell Biol.* 48:31; Pettitt et al., 1996, *Development* 122:4149-4157; Ramirez-Solis et al., 1993, *Methods Enzymol.* 225:855; and Thomas et al., 1987, *Cell* 51:503, each of which is incorporated herein by reference in its entirety for all purposes.

The "knockout" cells and animals of the invention include cells and animals in which one or several units of the endogenous telomerase enzyme complex have been deleted or inhibited. Reconstitution of telomerase activity will save the cell or animal from senescence or, for cancer cells, cell death caused by its inability to maintain telomeres. Methods of altering the expression of endogenous genes are well known to those of skill in the art. Typically, such methods involve altering or replacing all or a portion of the regulatory sequences controlling expression of the particular gene to be regulated. The regulatory sequences, e.g., the native promoter can be altered. The conventional technique for targeted mutation of genes involves placing a genomic DNA fragment containing the gene of interest into a vector, followed by cloning of the two genomic arms associated with the targeted gene around a selectable neomycin-resistance cassette in a vector containing thymidine kinase. This "knock-out" construct is then transfected into the appropriate host cell, i.e., a mouse embryonic stem (ES) cell, which is subsequently subjected to positive selection (using G418, for example, to select for neomycin-resistance) and negative selection (using, for example, FIAU to exclude cells lacking thymidine kinase), allowing the selection of cells which have undergone homologous recombination with the knockout vector. This approach leads to inactivation of the gene of interest. See, e.g., U.S. patents 5,464,764; 5,631,153; 5,487,992; and 5,627,059.

"Knocking" expression of an endogenous gene can also be accomplished by the use of homologous recombination to introduce a heterologous nucleic acid into the regulatory sequences (e.g., promoter) of the gene of interest. To

prevent expression of functional enzyme or product, simple mutations that either alter the reading frame or disrupt the promoter can be suitable. To up-regulate expression, a native promoter can be substituted with a heterologous promoter that induces higher levels of transcription. Also, "gene trap insertion" can be used to disrupt a host gene, and mouse ES cells can be used to produce knockout transgenic animals, as described for example, in Holzschu (1997) *Transgenic Res* 6: 97-106.

Altering the expression of endogenous genes by homologous recombination can also be accomplished by using nucleic acid sequences comprising the structural gene in question. Upstream sequences are utilized for targeting heterologous recombination constructs. Utilizing TRT structural gene sequence information, such as SEQUENCE ID NO:1, one of skill in the art can create homologous recombination constructs with only routine experimentation. Homologous recombination to alter expression of endogenous genes is described in U.S. Patent 5,272,071, and WO 91/09955, WO 93/09222, WO 96/29411, WO 95/31560, and WO 91/12650. Homologous recombination in mycobacteria is described by Azad (1996) *Proc Natl Acad Sci USA* 93:4787; Baulard (1996) *J. Bacteriol* 178:3091; and Pelicic (1996) *Mol. Microbiol* 20:919. Homologous recombination in animals has been described by Moynahan (1996) *Hum. Mol. Genet.* 5:875, and in plants by Offringa (1990) *EMBO J.* 9:3077.

XII. GLOSSARY

The following terms are defined *infra* to provide additional guidance to one of skill in the practice of the invention: adjuvant, allele (& allelic sequence), amino acids (including hydrophobic, polar, charged), conservative substitution, control elements (& regulatory sequences), derivatized, detectable label, elevated level, epitope, favorable and unfavorable prognosis, fusion protein, gene product, hTR, immortal, immunogen and immunogenic, isolated, modulator, motif, nucleic acid (& polynucleotide), oligonucleotides (& oligomers), operably linked, polypeptide, probe (including nucleic acid probes & antibody probes), recombinant, selection system, sequence, specific binding, stringent hybridization conditions (& stringency), substantial identity (& substantial similarity), substantially pure (& substantially

purified), telomerase-negative and telomerase-positive cells, telomerase catalytic activity, telomerase-related, and test compound.

As used herein, the term "adjuvant" refers to its ordinary meaning of
5 any substance that enhances the immune response to an antigen with which it is mixed. Adjuvants useful in the present invention include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. BCG (*Bacillus Calmette-Guerin*) and
10 *Corynebacterium parvum* are potentially useful adjuvants.

As used herein, the terms "allele" or "allelic sequence" refer to an alternative form of a nucleic acid sequence (i.e., a nucleic acid encoding hTERT protein). Alleles result from mutations (i.e., changes in the nucleic acid sequence), and generally
15 produce altered and/or differently regulated mRNAs or polypeptides whose structure and/or function may or may not be altered. Common mutational changes that give rise to alleles are generally ascribed to natural deletions, additions, or substitutions of nucleotides that may or may not affect the encoded amino acids. Each of these types of changes may occur alone, in combination with the others, or one or more times within a
20 given gene, chromosome or other cellular nucleic acid. Any given gene may have no, one or many allelic forms. As used herein, the term "allele" refers to either or both a gene or an mRNA transcribed from the gene.

As used herein, "amino acids" are sometimes specified using the
25 standard one letter code: Alanine (A), Serine (S), Threonine (T), Aspartic acid (D), Glutamic acid (E) Asparagine (N), Glutamine (Q), Arginine (R), Lysine (K), Isoleucine (I), Leucine (L), Methionine (M), Valine (V), Phenylalanine (F), Tyrosine (Y), Tryptophan (W), Proline (P), Glycine (G), Histidine (H), Cysteine (C). Synthetic and non-naturally occurring amino acid analogues (and/or peptide linkages) are included.

30

As used herein, "Hydrophobic amino acids" refers to A, L, I, V, P, F.

W, and M. As used herein, "polar amino acids" refers to G, S, T, Y, C, N, and Q. As used herein, "charged amino acids" refers to D, E, H, K, and R.

As used herein, "conservative substitution", when describing a protein refers to a change in the amino acid composition of the protein that does not substantially alter the protein's activity. Thus, "conservatively modified variations" of a particular amino acid sequence refers to amino acid substitutions of those amino acids that are not critical for protein activity or substitution of amino acids with other amino acids having similar properties (e.g., acidic, basic, positively or negatively charged, polar or non-polar, etc.) such that the substitutions of even critical amino acids does not substantially alter activity. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W) (see also, Creighton (1984) *Proteins*, W H. Freeman and Company). One of skill in the art will appreciate that the above-identified substitutions are not the only possible conservative substitutions. For example, one may regard all charged amino acids as conservative substitutions for each other whether they are positive or negative. In addition, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence can also be "conservatively modified variations". One can also make a "conservative substitution" in a recombinant protein by utilizing one or more codons that differ from the codons employed by the native or wild-type gene. In this instance, a conservative substitution also includes substituting a codon for an amino acid with a different codon for the same amino acid.

As used herein, "control elements" or "regulatory sequences" include enhancers, promoters, transcription terminators, origins of replication, chromosomal integration sequences, 5' and 3' untranslated regions, with which proteins or other

biomolecules interact to carry out transcription and translation. For eukaryotic cells, the control sequences will include a promoter and preferably an enhancer, e.g., derived from immunoglobulin genes, SV40, cytomegalovirus, and a polyadenylation sequence, and may include splice donor and acceptor sequences. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used

As used herein, a "derivatized" polynucleotide, oligonucleotide, or nucleic acid refers to oligo- and polynucleotides that comprise a derivatized substituent. In some embodiments, the substituent is substantially non-interfering with respect to hybridization to complementary polynucleotides. Derivatized oligo- or polynucleotides that have been modified with appended chemical substituents (e.g., by modification of an already synthesized oligo- or poly-nucleotide, or by incorporation of a modified base or backbone analog during synthesis) may be introduced into a metabolically active eukaryotic cell to hybridize with an hTERT DNA, RNA, or protein where they produce an alteration or chemical modification to a local DNA, RNA, or protein. Alternatively, the derivatized oligo or polynucleotides may interact with and alter hTERT polypeptides, telomerase-associated proteins, or other factors that interact with hTERT DNA or hTERT gene products, or alter or modulate expression or function of hTERT DNA, RNA or protein. Illustrative attached chemical substituents include: europium (III) texaphyrin, cross-linking agents, psoralen, metal chelates (e.g., iron/EDTA chelate for iron catalyzed cleavage), topoisomerases, endonucleases, exonucleases, ligases, phosphodiesterases, photodynamic porphyrins, chemotherapeutic drugs (e.g., adriamycin, doxorubicin), intercalating agents, base-modification agents, immunoglobulin chains, and oligonucleotides. Iron/EDTA chelates are chemical substituents often used where local cleavage of a polynucleotide sequence is desired (Hertzberg et al., 1982, *J. Am. Chem. Soc.* 104: 313; Hertzberg and Dervan, 1984, *Biochemistry* 23: 3934; Taylor et al., 1984, *Tetrahedron* 40: 457; Dervan, 1986, *Science* 232: 464. Illustrative attachment chemistries include: direct linkage, e.g., via an appended reactive amino group (Corey and Schultz (1988) *Science* 238: 1401, which is incorporated herein by reference) and other direct linkage chemistries, although

streptavidin/biotin and digoxigenin/anti-digoxigenin antibody linkage methods can also be used. Methods for linking chemical substituents are provided in U.S. Patents 5,135,720, 5,093,245, and 5,055,556, which are incorporated herein by reference. Other linkage chemistries may be used at the discretion of the practitioner.

5

As used herein, a "detectable label" has the ordinary meaning in the art and refers to an atom (e.g., radionuclide), molecule (e.g., fluorescein), or complex, that is or can be used to detect (e.g., due to a physical or chemical property), indicate the presence of a molecule or to enable binding of another molecule to which it is covalently bound or otherwise associated. The term "label" also refers to covalently bound or otherwise associated molecules (e.g., a biomolecule such as an enzyme) that act on a substrate to produce a detectable atom, molecule or complex. Detectable labels suitable for use in the present invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Labels useful in the present invention include biotin for staining with labeled streptavidin conjugate, magnetic beads (e.g., Dynabeads™), fluorescent dyes (e.g., fluorescein, Texas red, rhodamine, green fluorescent protein, enhanced green fluorescent protein, lissamine, phycoerythrin, Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7, FluorX [Amersham], SyBR Green I & II [Molecular Probes], and the like), radiolabels (e.g., ^3H , ^{32}P , ^{35}S , ^{14}C , or ^{125}I), enzymes (e.g., hydrolases, particularly phosphatases such as alkaline phosphatase, esterases and glycosidases, or oxidoreductases, particularly peroxidases such as horse radish peroxidase, and others commonly used in ELISAs), substrates, cofactors, inhibitors, chemiluminescent groups, chromogenic agents, and colorimetric labels such as colloidal gold or colored glass or plastic (e.g., polystyrene, polypropylene, latex, *etc.*) beads. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Means of detecting such labels are well known to those of skill in the art. Thus, for example, radiolabels and chemiluminescent labels may be detected using photographic film or scintillation counters, fluorescent markers may be detected using a photodetector to detect emitted light (e.g., as in fluorescence-activated cell sorting). Enzymatic labels are typically detected by providing the enzyme with a

30

substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and colorimetric labels are detected by simply visualizing the colored label. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (e.g., biotin) is covalently bound to the molecule. The ligand then binds to an anti-ligand (e.g., streptavidin) molecule which is either inherently detectable or covalently bound to a signal generating system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. A number of ligands and anti-ligands can be used. Where a ligand has a natural anti-ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labeled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody. The molecules can also be conjugated directly to signal generating compounds, e.g., by conjugation with an enzyme or fluorophore. Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter, photographic film as in autoradiography, or storage phosphor imaging. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Also, simple colorimetric labels may be detected by observing the color associated with the label. It will be appreciated that when pairs of fluorophores are used in an assay, it is often preferred that they have distinct emission patterns (wavelengths) so that they can be easily distinguished.

The phrase "elevated level" refers to an amount of hTERT gene product (or other specified substance or activity) in a cell that is elevated or higher than the

level in a reference standard, e.g., for diagnosis, the level in normal, telomerase-negative cells in an individual or in other individuals not suffering from the condition, and for prognosis, the level in tumor cells from a variety of grades or classes of, e.g., tumors.

5

As used herein, the term "epitope" has its ordinary meaning of a site on an antigen recognized by an antibody. Epitopes are typically segments of amino acids which are a small portion of the whole protein. Epitopes may be conformational (*i.e.*, discontinuous). That is, they may be formed from amino acids encoded by noncontiguous parts of a primary sequence that have been juxtaposed by protein folding.

The terms "favorable prognosis" and "unfavorable prognosis" are known in the art. In the context of cancers, "favorable prognosis" means that there is a likelihood of tumor regression or longer survival times for patients with a favorable prognosis relative to those with unfavorable prognosis, whereas "unfavorable prognosis" means that the tumor is likely to be more aggressive, *i.e.*, grow faster and/or metastasize, resulting in a poor outcome or a more rapid course of disease progression for the patient.

20

As used herein, the term "fusion protein," refers to a composite protein, *i.e.*, a single contiguous amino acid sequence, made up of two (or more) distinct, heterologous polypeptides which are not normally fused together in a single amino acid sequence. Thus, a fusion protein may include a single amino acid sequence that contains two entirely distinct amino acid sequences or two similar or identical polypeptide sequences, provided that these sequences are not normally found together in the same configuration in a single amino acid sequence found in nature. Fusion proteins may generally be prepared using either recombinant nucleic acid methods, *i.e.*, as a result of transcription and translation of a recombinant gene fusion product, which fusion comprises a segment encoding a polypeptide of the invention and a segment encoding a heterologous protein, or by chemical synthesis methods well known in the

30

art. The non-hTRT region(s) of the fusion protein can be fused to the amino terminus of the hTRT polypeptide or the carboxyl terminus, or both or the non-hTRT region can be inserted into the interior of the protein sequence (by moiety inserting or by replacing amino acids) or combinations of the foregoing can be performed.

5

As used herein, the term "gene product" refers to an RNA molecule transcribed from a gene, or a protein encoded by the gene or translated from the RNA.

As used herein, "hTR" (human telomerase RNA) refers to the RNA component of human telomerase and any naturally occurring alleles and variants or recombinant variants. hTR is described in detail in U.S. Patent No. 5,583,016 which is incorporated herein by reference in its entirety and for all purposes.

As used herein, the term "immortal," when referring to a cell, has its normal meaning in the telomerase art and refers to cells that have apparently unlimited replicative potential. Immortal can also refer to cells with increased proliferative capacity relative to their unmodified counterparts. Examples of immortal human cells are malignant tumor cells, germ line cells, and certain transformed human cell lines cultured *in vitro* (e.g., cells that have become immortal following transformation by viral oncogenes or otherwise). In contrast, most normal human somatic cells are mortal, i.e., have limited replicative potential and become senescent after a finite number of cell divisions.

As used herein, the terms "immunogen" and "immunogenic" have their ordinary meaning in the art, i.e., an immunogen is a molecule, such as a protein or other antigen, that can elicit an adaptive immune response upon injection into a person or an animal.

As used herein, "isolated," when referring to a molecule or composition, such as, for example, an RNP (e.g., at least one protein and at least one RNA), means that the molecule or composition is separated from at least one other compound, such as

a protein, other RNAs, or other contaminants with which it is associated *in vivo* or in its naturally occurring state. Thus, an RNP is considered isolated when the RNP has been isolated from any other component with which it is naturally associated, e.g., cell membrane, as in a cell extract. An isolated composition can, however, also be substantially pure.

As used herein, "modulator" refers to any synthetic or natural compound or composition that can change in any way either or both the "full" or any "partial activity" of a telomerase reverse transcriptase (TRT). A modulator can be an agonist or an antagonist. A modulator can be any organic and inorganic compound; including, but not limited to, for example, small molecules, peptides, proteins, sugars, nucleic acids, fatty acids and the like.

As used herein, "motif" refers to a sequence of contiguous amino acids (or to a nucleic acid sequence that encodes a sequence of contiguous amino acids) that defines a feature or structure in a protein that is common to or conserved in all proteins of a defined class or type. The motif or consensus sequence may include both conserved and non-conserved residues. The conserved residues in the motif sequence indicate that the conserved residue or class (i.e., hydrophobic, polar, non-polar, or other class) of residues is typically present at the indicated location in each protein (or gene or mRNA) of the class of proteins defined by the motif. Motifs can differ in accordance with the class of proteins. Thus, for example, the reverse transcriptase enzymes form a class of proteins that can be defined by one or more motifs, and this class includes telomerase enzymes. However, the telomerase enzymes can also be defined as the class of enzymes with motifs characteristic for that class. Those of skill recognize that the identification of a residue as a conserved residue in a motif does not mean that every member of the class defined by the motif has the indicated residue (or class of residues) at the indicated position, and that one or more members of the class may have a different residue at the conserved position.

30

As used herein, the terms "nucleic acid" and "polynucleotide" are used

interchangeably. Use of the term "polynucleotide" is not intended to exclude oligonucleotides (i.e., short polynucleotides) and can also refer to synthetic and/or non-naturally occurring nucleic acids (i.e., comprising nucleic acid analogues or modified backbone residues or linkages).

5

As used herein "oligonucleotides" or "oligomers" refer to a nucleic acid sequence of approximately 7 nucleotides or greater, and as many as approximately 100 nucleotides, which can be used as a primer, probe or amplimer. Oligonucleotides are often between about 10 and about 50 nucleotides in length, more often between about 14 and about 35 nucleotides, very often between about 15 and about 25 nucleotides, and the terms oligonucleotides or oligomers can also refer to synthetic and/or non-naturally occurring nucleic acids (i.e., comprising nucleic acid analogues or modified backbone residues or linkages).

15

As used herein, the term "operably linked," refers to a functional relationship between two or more nucleic acid (e.g., DNA) segments: for example, a promoter or enhancer is operably linked to a coding sequence if it stimulates the transcription of the sequence in an appropriate host cell or other expression system. Generally, sequences that are operably linked are contiguous, and in the case of a signal sequence both contiguous and in reading phase. However, enhancers need not be located in close proximity to the coding sequences whose transcription they enhance.

20

As used herein, the term "polypeptide" is used interchangeably herein with the term "protein," and refers to a polymer composed of amino acid residues linked by amide linkages, including synthetic, naturally-occurring and non-naturally occurring analogs thereof (amino acids and linkages). Peptides are examples of polypeptides.

25

As used herein, a "probe" refers to a molecule that specifically binds another molecule. One example of a probe is a "nucleic acid probe" that specifically binds (i.e., anneals or hybridizes) to a substantially complementary nucleic acid.

30

Another example of a probe is an "antibody probe" that specifically binds to a corresponding antigen or epitope.

As used herein, "recombinant" refers to a polynucleotide synthesized or otherwise manipulated *in vitro* (e.g., "recombinant polynucleotide"), to methods of using recombinant polynucleotides to produce gene products in cells or other biological systems, or to a polypeptide ("recombinant protein") encoded by a recombinant polynucleotide.

As used herein, a "selection system," in the context of stably transformed cell lines, refers to a method for identifying and/or selecting cells containing a recombinant nucleic acid of interest. A large variety of selection systems are known for identification of transformed cells and are suitable for use with the present invention. For example, cells transformed by plasmids or other vectors can be selected by resistance to antibiotics conferred by genes contained on the plasmids, such as the well known *amp*, *gpt*, *neo* and *hyg* genes, or other genes such as the herpes simplex virus thymidine kinase (Wigler et al., *Cell* 11:223-32 [1977]) and adenine phosphoribosyltransferase (Lowy et al., *Cell* 22:817 [1980]) genes which can be employed in *tk-* or *aprt-* cells, respectively. Also, antimetabolite, antibiotic or herbicide resistance can be used as the basis for selection; for example, *dhfr* which confers resistance to methotrexate and is also useful for gene amplification (Wigler et al., *Proc. Natl. Acad. Sci.*, 77:3567 [1980]); *npt*, which confers resistance to the aminoglycosides neomycin and G-418 (Colbere-Garapin et al., *J. Mol. Biol.*, 150:1 [1981]) and *als* or *pat*, which confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murry, in McGraw Hill Yearbook of Science and Technology, McGraw Hill, New York NY, pp 191-196, [1992]). Additional selectable genes have been described, for example, hygromycin resistance-conferring genes, *trpB*, which allows cells to utilize indole in place of tryptophan, or *hisD*, which allows cells to utilize histinol in place of histidine (Hartman and Mulligan, *Proc. Natl. Acad. Sci.*, 85:8047 [1988]). Recently, the use of visible markers has gained popularity with such markers as anthocyanins, beta-glucuronidase and its substrate, GUS, and luciferase and its

substrate, luciferin, being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes et al., *Meth. Mol. Biol.*, 55:121 [1995]).

5 As used herein, the "sequence" of a gene (unless specifically stated otherwise), nucleic acid, protein, or peptide refers to the order of nucleotides in either or both strands of a double-stranded DNA molecule, e.g., the sequence of both the coding strand and its complement, or of a single-stranded nucleic acid molecule, or to the order of amino acids in a peptide or protein.

10 As used herein, "specific binding" refers to the ability of one molecule, typically an antibody or polynucleotide, to contact and associate with another specific molecule even in the presence of many other diverse molecules. For example, a single-stranded polynucleotide can specifically bind to a single-stranded polynucleotide that is
15 complementary in sequence, and an antibody specifically binds to (or "is specifically immunoreactive with") its corresponding antigen.

 As used herein, "stringent hybridization conditions" or "stringency" refers to conditions in a range from about 5°C to about 20°C or 25°C below the
20 melting temperature (T_m) of the target sequence and a probe with exact or nearly exact complementarity to the target. As used herein, the melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half-dissociated into single strands. Methods for calculating the T_m of nucleic acids are well known in the art (see, e.g., Berger and Kimmel (1987) *METHODS IN ENZYMOLOGY*,
25 VOL. 152: *GUIDE TO MOLECULAR CLONING TECHNIQUES*, San Diego: Academic Press, Inc. and Sambrook et al. (1989) *MOLECULAR CLONING: A LABORATORY MANUAL*, 2ND ED., VOLS. 1-3, Cold Spring Harbor Laboratory hereinafter, "Sambrook"), both incorporated herein by reference). As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\% G +$
30 C), when a nucleic acid is in aqueous solution at 1 M NaCl (see e.g., Anderson and Young, *Quantitative Filter Hybridization* in *NUCLEIC ACID HYBRIDIZATION* (1985)).

Other references include more sophisticated computations which take structural as well as sequence characteristics into account for the calculation of T_m . The melting temperature of a hybrid (and thus the conditions for stringent hybridization) is affected by various factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, and the like), and the concentration of salts and other components (e.g., the presence or absence of formamide, dextran sulfate, polyethylene glycol). The effects of these factors are well known and are discussed in standard references in the art, e.g., Sambrook, *supra* and Ausubel et al. *supra*. Typically, stringent hybridization conditions are salt concentrations less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion at pH 7.0 to 8.3, and temperatures at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). As noted, stringent conditions may also be achieved with the addition of destabilizing agents such as formamide, in which case lower temperatures may be employed.

As used herein, the term "substantial identity," "substantial sequence identity," or "substantial similarity" in the context of nucleic acids, refers to a measure of sequence similarity between two polynucleotides. Substantial sequence identity can be determined by hybridization under stringent conditions, by direct comparison, or other means. For example, two polynucleotides can be identified as having substantial sequence identity if they are capable of specifically hybridizing to each other under stringent hybridization conditions. Other degrees of sequence identity (e.g., less than "substantial") can be characterized by hybridization under different conditions of stringency. Alternatively, substantial sequence identity can be described as a percentage identity between two nucleotide (or polypeptide) sequences. Two sequences are considered substantially identical when they are at least about 60% identical, preferably at least about 70% identical, or at least about 80% identical, or at least about 90% identical, or at least about 95% or 98% to 100% identical. Percentage sequence (nucleotide or amino acid) identity is typically calculated by determining the optimal alignment between two sequences and comparing the two sequences. For

example an exogenous transcript used for protein expression can be described as having a certain percentage of identity or similarity compared to a reference sequence (e.g., the corresponding endogenous sequence). Optimal alignment of sequences may be conducted using the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection. The best alignment (i.e., resulting in the highest percentage of identity) generated by the various methods is selected. Typically these algorithms compare the two sequences over a "comparison window" (usually at least 18 nucleotides in length) to identify and compare local regions of sequence similarity, thus allowing for small additions or deletions (i.e., gaps). Additions and deletions are typically 20 percent or less of the length of the sequence relative to the reference sequence, which does not comprise additions or deletions. It is sometimes desirable to describe sequence identity between two sequences in reference to a particular length or region (e.g., two sequences may be described as having at least 95% identity over a length of at least 500 basepairs). Usually the length will be at least about 50, 100, 200, 300, 400 or 500 basepairs, amino acids, or other residues. The percentage of sequence identity is calculated by comparing two optimally aligned sequences over the region of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, or U) occurs in both sequences to yield the number of matched positions, and determining the number (or percentage) of matched positions as compared to the total number of bases in the reference sequence or region of comparison. An additional algorithm that is suitable for determining sequence similarity is the BLAST algorithm, which is described in Altschul (1990) *J. Mol. Biol.* 215: 403-410; and Shpaer (1996) *Genomics* 38:179-191. Software for performing BLAST analyses is publicly available at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query

sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al, supra.*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (see Henikoff (1992) *Proc. Natl. Acad. Sci. USA* 89: 10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands. The term BLAST refers to the BLAST algorithm which performs a statistical analysis of the similarity between two sequences: see, e.g., Karlin (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5787. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid can be considered similar to a TRT nucleic acid if the smallest sum probability in a comparison of the test nucleic acid to an TRT nucleic acid is less than about 0.5, 0.2, 0.1, 0.01, or 0.001. Alternatively, another indication that two nucleic acid sequences are similar is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

As used herein, the terms "substantial identity," "substantial sequence identity," or "substantial similarity" in the context of a polypeptide, refers to a degree of similarity between two polypeptides in which a polypeptides comprises a sequence with at least 70% sequence identity to a reference sequence, or 80%, or 85% or up to 100% sequence identity to the reference sequence, or most preferably 90% identity over

a comparison window of about 10-20 amino acid residues. Amino acid sequence similarity, or sequence identity, is determined by optimizing residue matches, if necessary, by introducing gaps as required. See Needleham et al. (1970) *J. Mol. Biol.* 48: 443-453; and Sankoff et al., 1983, *Time Warps, String Edits, and Macromolecules*,
5 *The Theory and Practice of Sequence Comparison*, Chapter One, Addison-Wesley, Reading, MA; and software packages from IntelliGenetics, Mountain View, CA, and the University of Wisconsin Genetics Computer Group, Madison, WI. As will be apparent to one of skill, the terms "substantial identity", "substantial similarity" and "substantial sequence identity" can be used interchangeably with regard to polypeptides
10 or polynucleotides.

As used herein, the term "substantially pure," or "substantially purified," when referring to a composition comprising a specified reagent, such as an antibody (e.g. an anti-hTERT antibody), means that the specified reagent is at least about
15 75%, or at least about 90%, or at least about 95%, or at least about 99% or more of the composition (not including, e.g., solvent or buffer). Thus, for example, a preferred immunoglobulin preparation of the invention that specifically binds an hTERT polypeptide is substantially purified.

20 As used herein, a "telomerase negative" cell is one in which telomerase is not expressed, i.e., no telomerase catalytic activity can be detected using a conventional assay or a TRAP assay for telomerase catalytic activity. As used herein, a "telomerase positive" cell is a cell in which telomerase is expressed (i.e. telomerase activity can be detected).

25 As used herein, a "telomerase-related" disease or condition is a disease or condition in a subject that is correlated with an abnormally high level of telomerase activity in cells of the individual, which can include any telomerase activity at all for most normal somatic cells, or which is correlated with a low level of telomerase activity
30 that results in impairment of a normal cell function. Examples of telomerase-related conditions include, e.g., cancer (high telomerase activity in malignant cells) and

infertility (low telomerase activity in germ-line cells).

As used herein, "test compound" or "agent" refers to any synthetic or natural compound or composition. The term includes all organic and inorganic compounds; including, for example, small molecules, peptides, proteins, sugars, nucleic acids, fatty acids and the like.

XIII. EXAMPLES

The following examples are provided to illustrate the present invention, and not by way of limitation.

In the following sections, the following abbreviations apply: eq (equivalents); M (Molar); μ M (micromolar); N (Normal); mol (moles); mmol (millimoles); μ mol (micromoles); nmol (nanomoles); g (grams); mg (milligrams); μ g (micrograms); ng (nanograms); l or L (liters); ml (milliliters); μ l (microliters); cm (centimeters); mm (millimeters); μ m (micrometers); nm (nanometers); $^{\circ}$ C (degrees Centigrade); RPN (ribonucleoprotein); m²EN (2'-O-methylribonucleotides); dNTP (deoxyribonucleotides); dH₂O (distilled water); DDT (dithiothreitol); PMSF (phenylmethylsulfonyl fluoride); TE (10 mM Tris HCl, 1 mM EDTA, approximately pH 7.2); KGlu (potassium glutamate); SSC (salt and sodium citrate buffer); SDS (sodium dodecyl sulfate); PAGE (polyacrylamide gel electrophoresis); Novex (Novex, San Diego, CA); BioRad (Bio-Rad Laboratories, Hercules, CA); Pharmacia (Pharmacia Biotech, Piscataway, NJ); Boehringer-Mannheim (Boehringer-Mannheim Corp., Concord, CA); Amersham (Amersham, Inc., Chicago, IL); Stratagene (Stratagene Cloning Systems, La Jolla, CA); NEB (New England Biolabs, Beverly, MA); Pierce (Pierce Chemical Co., Rockford, IL); Beckman (Beckman Instruments, Fullerton, CA); Lab Industries (Lab Industries, Inc., Berkeley, CA); Eppendorf (Eppendorf Scientific, Madison, WI); and Molecular Dynamics (Molecular Dynamics, Sunnyvale, CA).

EXAMPLE 1

ISOLATION OF TELOMERASE PROTEINS AND CLONES

The following example details the isolation of telomerase proteins and clones from various organisms, including the eukaryotes p. 123, hTERT, TRT and *S.*

5 *pombe* TRT telomerase cDNA clones.

A. Background

i) Introduction

This section provides an overview of the purification and cloning of TRT genes, which is described in greater detail in subsequent sections of this Example.

10 While telomerase RNA subunits have been identified in ciliates, yeast and mammals, protein subunits of the enzyme have not been identified as such prior to the present invention. Purification of telomerase from the ciliated protozoan *Euplotes aediculatus* yielded two proteins, termed p123 and p43 (see *infra*; Lingner (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93:10712). *Euplotes aediculatus* is a hypotrichous ciliate having a
15 macronucleus containing about 8×10^7 telomeres and about 3×10^5 molecules of telomerase. After purification, the active telomerase complex had a molecular mass of about 230 kD, corresponding to a 66 kD RNA subunit and two proteins of about 123 kD and 43 kD (Lingner (1996) *supra*). Photocross-linking experiments indicated that the larger p123 protein was involved in specific binding of the telomeric DNA substrate
20 (Lingner, (1996) *supra*).

The p123 and p43 proteins were sequenced and the cDNA clones which encoded these proteins were isolated. These *Euplotes* sequences were found to be unrelated to the *Tetrahymena* telomerase-associated proteins p80 and p95. Sequence analysis of the *Euplotes* p123 revealed reverse transcriptase (RT) motifs. Furthermore,
25 sequence analysis of the *Euplotes* p123 by comparison to other sequences revealed a yeast homolog, termed Est2 protein (Lingner (1997) *Science* 276:561). Yeast Est2 had previously been shown to be essential for telomere maintenance *in vivo* (Lendvay (1996) *Genetics* 144:1399) but had not been identified as a telomerase catalytic protein. Site-specific mutagenesis demonstrated that the RT motifs of yeast Est2 are essential
30 for telomeric DNA synthesis *in vivo* and *in vitro* (Lingner (1997) *supra*).

ii) Identifying and Characterizing *S. pombe* Telomerase

PCR amplification of *S. pombe* DNA was carried out with degenerate sequence primers designed from the *Euplotes* p123 RT motifs as described below. Of the four prominent PCR products generated, a 120 base pair band encoded a peptide sequence homologous to p123 and Est2. This PCR product was used as a probe in colony hybridization and identified two overlapping clones from an *S. pombe* genomic library and three from an *S. pombe* cDNA library. Sequence analysis revealed that none of the three *S. pombe* cDNA clones was full length, so RT-PCR was used to obtain the sequences encoding the protein's N-terminus.

Complete sequencing of these clones revealed a putative *S. pombe* telomerase RT gene, *trt1*. The complete nucleotide sequence of *trt1* has been deposited in GenBank, accession number AF015783 (see Figure 15).

To test *S. pombe trt1* (as a catalytic subunit, two deletion constructs were created. Analysis of the sequence showed that *trt1* encoded a basic protein with a predicted molecular mass of 116 kD. It was found that homology with p123 and Est2 was especially high in the seven reverse transcriptase motifs, underlined and designated as motifs 1, 2, A, B, C, D, and E (see Figure 63). An additional telomerase-specific motif, designated the T-motif, was also found. Fifteen introns, ranging in size from 36 to 71 base pairs, interrupted the coding sequence.

To test *S. pombe trt1* as a catalytic subunit, two deletion constructs were created. One removed only motifs B through D in the RT domains. The second removed 99% of the open reading frame.

Haploid cells grown from *S. pombe* spores of both mutants showed progressive telomere shortening to the point where hybridization to telomeric repeats became almost undetectable. A *trt1⁻/trt1⁻* diploid was sporulated and the resulting tetrads were dissected and germinated on a yeast extract medium supplemented with amino acids (a YES plate, Alfa (1993) *Experiments with Fission Yeast*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Colonies derived from each spore were grown at 32°C for three days, and streaked successively to fresh YES plates every three days. A colony from each round was placed in six ml of YES liquid culture at 32°C and grown to stationary phase. Genomic DNA was prepared. After digestion with *Apal*, DNA was subjected to electrophoresis on a 2.3% agarose gel, stained with

ethidium bromide to confirm approximately equal loading in each lane, then transferred to a nylon membrane and hybridized to a telomeric DNA probe.

Senescence was indicated by the delayed onset of growth or failure to grow on agar (typically at the fourth streak-out after germination) and by colonies with increasingly ragged edges (colony morphology shown in Figure 22C) and by increasingly high fractions of elongated cells (as shown in Figure 22D). Cells were plated on Minimal Medium (Alfa (1993) *supra*) with glutamic acid substituted for ammonium chloride for two days at 32°C prior to photography.

When individual enlarged cells were separated on the dissecting microscope, the majority were found to undergo no further division. The same telomerase negative (*trt1*⁻) cell population always contained normal-sized cells which continued to divide, but which frequently produced non-dividing progeny. The telomerase-negative survivors may use a recombinational mode of telomere maintenance as documented in budding yeast strains that have various telomere-replication genes deleted (Lendvay (1996) *supra*, Lundblad (1993) *Cell* 73:347).

iii) Identifying and Characterizing Human Telomerase

An EST (expressed sequence tag) derived from human telomerase reverse transcriptase (hTRT) cDNA was identified by a BLAST search of the dbEST (expressed sequence tag) Genbank database using the *Euplotes* 123 kDa peptide and nucleic acid sequences, as well as the *Schizosaccharomyces* protein and corresponding cDNA (*tez1*) sequences. The EST, designated Genbank AA28196, is 389 nucleotides long and it corresponds to positions 1679 to 2076 of clone 712562 (Figure 18), was obtained from the I.M.A.G.E. Consortium (Human Genome Center, DOE, Lawrence Livermore National Laboratory, Livermore, CA). This clone was obtained from a cDNA library of germinal B cells derived by flow sorting of tonsil cells. Complete sequencing of this hTRT cDNA clone showed all eight telomerase RT (TRT) motifs. However, this hTRT clone did not encode a contiguous portion of a TRT because RT motifs B, C, D, and E, were contained in a different open reading frame than the more N-terminal RT motifs. In addition, the distance between RT motifs A and B was substantially shorter than that of the three previously known (non-human) TRTs.

To isolate a full length cDNA clone, a cDNA library derived from the

human 293 cell line (described above) which expresses high levels of telomerase activity, was screened. A lambda cDNA library from the 293 cell line was partitioned into 25 pools containing about 200,000 plaques each. Each pool was screened by PCR with the primer pair 5'-CGGAAGAGTGTCTGGAGCAA-3' and 5'-GGATGAAGCGGAGTCTGGA-3'. Six subpools of one positive primary pool were further screened by PCR using this same primer pair. For both the primary and the secondary subpool screening, hTERT was amplified for a total of 31 cycles at: 94°C, 45 seconds; 60°C, 45 seconds; and 72°C, 90 seconds. As a control, RNA of the house-keeping enzyme GAPDH was amplified using the primer pair 5'-CTCAGACACCA TGGGGAAGGTGA-3' and 5'-ATGATCTTGAGGCTGTTGTCATA-3' for a total of 16 cycles at 94°C, 45 seconds; 55°C, 45 seconds; and 72°C, 90 seconds.

One hTERT positive subpool from the secondary screening was then screened by plaque hybridization with a probe from the 5' region of clone #712562. One phage was positively identified (designated Lambda phage 25-1.1, ATCC 209024, deposited May 12, 1997). It contained an approximately four kilobase insert, which was excised and subcloned into the EcoRI site of pBluescript II SK- vector (Stratagene, San Diego, CA) as an EcoRI fragment. This cDNA clone-containing plasmid was designated pGRN121. The cDNA insert totals approximately 4 kilobasepairs. The complete nucleotide sequence of the human hTERT cDNA (pGRN121) has been deposited in Genbank (accession AF015950) and the plasmid has been deposited with the ATCC (ATCC 209016, deposited May 6, 1997).

B. Growth of *Euplotes aediculatus*

In this Example, cultures of *E. aediculatus* were obtained from Dr. David Prescott, MCDB, University of Colorado. Dr. Prescott originally isolated this culture from pond water, although this organism is also available from the ATCC (ATCC #30859). Cultures were grown as described by Swanton *et al.* (Swanton *et al.*, Chromosoma 77:203 [1980]), under non-sterile conditions, in 15-liter glass containers containing *Chlorogonium* as a food source. Organisms were harvested from the cultures when the density reached approximately 10⁴ cells/ml.

C. Preparation of Nuclear Extracts

In this Example, nuclear extracts of *E. aediculatus* were prepared using the method of Lingner *et al.* (Lingner *et al.*, Genes Develop., 8:1984 [1994]), with minor modifications, as indicated below. Briefly, cells grown as described in Part B were concentrated with 15 μ m Nytex filters and cooled on ice. The cell pellet was resuspended in a final volume of 110 ml TMS/PMSF/spermidine phosphate buffer. The stock TMS/PMSF/spermidine phosphate buffer was prepared by adding 0.075 g spermidine phosphate (USB) and 0.75 ml PMSF (from 100 mM stock prepared in ethanol) to 150 ml TMS. TMS comprised 10 mM Tris-acetate, 10 mM $MgCl_2$, 85.5752 g sucrose/liter, and 0.33297 g $CaCl_2$ /liter, pH 7.5.

After resuspension in TMS/PMSF/spermidine phosphate buffer, 8.8 ml 10% NP-40 and 94.1 g sucrose were added and the mixture placed in a siliconized glass beaker with a stainless steel stirring rod attached to an overhead motor. The mixture was stirred until the cells were completely lysed (approximately 20 minutes). The mixture was then centrifuged for 10 minutes at 7500 rpm (8950 x g), at 4°C, using a Beckman JS-13 swing-out rotor. The supernatant was removed and nuclei pellet was resuspended in TMS/PMSF/spermidine phosphate buffer, and centrifuged again, for 5 minutes at 7500 rpm (8950 x g), at 4°C, using a Beckman JS-13 swing-out rotor.

The supernatant was removed and the nuclei pellet was resuspended in a buffer comprised of 50 mM Tris-acetate, 10 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 0.4 M K₂Glu, 0.5 mM PMSF, pH 7.5, at a volume of 0.5 ml buffer per 10 g of harvested cells. The resuspended nuclei were then dounced in a glass homogenizer with approximately 50 strokes, and then centrifuged for 25 minutes at 14,000 rpm at 4°C, in an Eppendorf centrifuge. The supernatant containing the nuclear extract was collected, frozen in liquid nitrogen, and stored at -80°C until used.

D. Purification of Telomerase

In this Example, nuclear extracts prepared as described in Part C were used to purify *E. aediculatus* telomerase. In this purification protocol, telomerase was first enriched by chromatography on an Affi-Gel-heparin column, and then extensively purified by affinity purification with an antisense oligonucleotide. As the template

region of telomerase RNA is accessible to hybridization in the telomerase RNP particle, an antisense oligonucleotide (*i.e.*, the "affinity oligonucleotide") was synthesized that was complementary to this template region as an affinity bait for the telomerase. A biotin residue was included at the 5' end of the oligonucleotide to immobilize it to an
5 avidin column.

Following the binding of the telomerase to the oligonucleotide, and extensive washing, the telomerase was eluted by use of a displacement oligonucleotide. The affinity oligonucleotide included DNA bases that were not complementary to the telomerase RNA 5' to the telomerase-specific sequence. As the displacement
10 oligonucleotide was complementary to the affinity oligonucleotide for its entire length, it was able to form a more thermodynamically stable duplex than the telomerase bound to the affinity oligonucleotide. Thus, addition of the displacement oligonucleotide resulted in the elution of the telomerase from the column.

The nuclear extracts prepared from 45 liter cultures were frozen until a
15 total of 34 ml of nuclear extract was collected. This corresponded to 630 liters of culture (*i.e.*, approximately 4×10^7 cells). The nuclear extract was diluted with a buffer to 410 ml, to provide final concentrations of 20 mM Tris-acetate, 1 mM $MgCl_2$, 0.1 mM EDTA, 33 mM KGlu, 10% (vol/vol) glycerol, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), at a pH of 7.5.

20 The diluted nuclear extract was applied to an Affi-Gel-heparin gel column (Bio-Rad), with a 230 ml bed volume and 5 cm diameter, equilibrated in the same buffer and eluted with a 2-liter gradient from 33 to 450 mM KGlu. The column was run at 4°C, at a flow rate of 1 column volume/hour. Fractions of 50 mls each were collected and assayed for telomerase activity as described in Part E. Telomerase was
25 eluted from the column at approximately 170 mM KGlu. Fractions containing telomerase (approximately 440 ml) were pooled and adjusted to 20 mM Tris-acetate, 10 mM $MgCl_2$, 1 mM EDTA, 300 mM KGlu, 10% glycerol, 1 mM DTT, and 1% Nonidet P-40. This buffer was designated as "WB."

To this preparation, 1.5 nmol of each of two competitor DNA
30 oligonucleotides (5'-TAGACCTGTTAGTGTACATTTGAATTGAAGC-3' (and 5'-TAGACCTGTTAGGTTGGATTGTGGCATCA-3', 50 µg yeast RNA (Sigma), and

0.3 nmol of biotin-labeled telomerase-specific oligonucleotide (5'-biotin-TAGACCTGTTA-(mreG)₂-(rmeU)₄-(rmeG)₄-(rmeU)₄-remG-3'), were added per ml of the pool. The 2-O-methyribonucleotides of the telomerase specific oligonucleotides were complementary to the the telomerase RNA; template region; the
5 deoxyribonucleotides were not complementary. The inclusion of competitor, non-specific DNA oligonucleotides increased the efficiency of the purification, as the effects of nucleic acid binding proteins and other components in the mixture that would either bind to the affinity oligonucleotide or remove the telomerase from the mixture were minimized.

10 This material was then added to Ultralink immobilized neutravidin plus (Pierce) column material, at a volume of 60 µl of suspension per ml of pool. The column material was pre-blocked twice for 15 minutes each blocking, with a preparation of WB containing 0.01% Nonidet P-40, 0.5 mg BSA, 0.5 mg/ml lysozyme, 0.05 mg/ml glycogen, and 0.1 mg/ml yeast RNA. The blocking was conducted at 4°C,
15 using a rotating wheel to block the column material thoroughly. After the first blocking step, and before the second blocking step, the column material was centrifuged at 200 x g for 2 minutes to pellet the matrix

The pool-column mixture was incubated for 8 minutes at 30°C, and then for an additional 2 hours at 4°C, on a rotating wheel (approximately 10 rpm;
20 Labindustries) to allow binding. The pool-column mixture was then centrifuged 200 xg for 2 minutes, and the supernatant containing unbound material was removed. The pool-column mixture was then washed. This washing process included the steps of rinsing the pool-column mixture with WB at 4°C, washing the mixture for 15 minutes with WB at 4°C, rinsing with WB, washing for 5 minutes at 30°C, with WB containing
25 0.6 M KGlu, and no Nonidet P-40, washing 5 minutes at 25°C with WB, and finally, rinsing again with WB. The volume remaining after the final wash was kept small, in order to yield a ratio of buffer to column material of approximately 1:1.

Telomerase was eluted from the column material by adding 1 nmol of displacement deoxyoligonucleotide (5'-CA₂C₄A₄C₂TA₂CAG₂TCTA-3'), per ml of
30 column material and incubating at 25°C for 30 minutes. The material was centrifuged for 2 minutes at 14,000 rpm in a microcentrifuge (Eppendorf), and the eluate collected.

The elution procedure was repeated twice more, using fresh displacement oligonucleotide each time. As mentioned above, because the displacement oligonucleotide was complementary to the affinity oligonucleotide, it formed a more thermodynamically stable complex with the affinity oligonucleotide than P-40. Thus, addition of the displacement oligonucleotide to an affinity-bound telomerase resulted in efficient elution of telomerase under native conditions. The telomerase appeared to be approximately 50% pure at this stage, as judged by analysis on a protein gel. The affinity purification of telomerase and elution with a displacement oligonucleotide is shown in Figure 26 (panels A and B, respectively). In this Figure, the 2'-O-methyl sugars of the affinity oligonucleotide are indicated by the bold line. The black and shaded oval shapes in this Figure are intended to represent graphically the protein subunits of the present invention.

The protein concentrations of the extract and material obtained following Affi-Gel-heparin column chromatography were determined using the method of Bradford (Bradford, Anal. Biochem., 72:248 [1976]), using BSA as the standard. Only a fraction of the telomerase preparation was further purified on a glycerol gradient.

The sedimentation coefficient of telomerase was determined by glycerol gradient centrifugation, as described in Part I.

Table 5 below is a purification table for telomerase purified according to the methods of this Example. The telomerase was enriched 12-fold in nuclear extracts, as compared to whole cell extracts, with a recovery of 80%; 85% of telomerase was solubilized from nuclei upon extraction.

Table 5. Purification of Telomerase

| Fraction | Protein (mg) | Telomerase (pmol of RNP) | Telomerase/ Protein/pmol of RNP/mg | Recovery (%) | Purification Factor |
|----------------------|--------------|--------------------------------|--|-----------------|------------------------|
| Nuclear Extract | 2020 | 1720 | 0.9 | 100 | 1 |
| Heparin | 125 | 1040 | 8.3 | 60 | 10 |
| Affinity | 0.3** | 680 | 2270 | 40 | 2670 |
| Glycerol Gradient | NA* | NA* | NA* | 25 | NA* |

*NA=Not available

**This value was calculated from the measured amount of telomerase (680 pmol), by assuming a purity of 50% (based on a protein gel).

B. Telomerase Activity

At each step in the purification of telomerase, the preparation was analyzed by three separate assays, one of which was activity, as described in this Example. In general, telomerase assays were done in 40 μ l containing 0.003-0.3 μ l of nuclear extract, 50 mM Tris-Cl (pH 7.5), 50 mM KGlu, 10 mM MgCl₂, 1 mM DTT, 125 μ M dTTP, 125 μ M dGTP, and approximately 0.2 pmoles of 5'-³²P-labelled oligonucleotide substrate (*i.e.*, approximately 400,000 cpm). Oligonucleotide primers were heat-denatured prior to their addition to the reaction mixture. Reactions were assembled on ice and incubated for 30 minutes at 25°C. The reactions were stopped by addition of 200 μ l of 10 mM Tris-Cl (pH 7.5), 15 mM EDTA, 0.6% SDS, and 0.05 mg/ml proteinase K, and incubated for at least 30 minutes at 45°C. After ethanol precipitation, the products were analyzed on denaturing 8% PAGE gels, as known in the art (*See e.g.*, Sambrook *et al.*, 1989).

F. Quantitation of Telomerase Activity

In this Example, quantitation of telomerase activity through the purification procedure is described. Quantitation was accomplished by assaying the elongation of oligonucleotide primers in the presence of dGTP and [α - 32 P]dTTP.

5 Briefly, 1 μ M 5'-(G₄T₄)₂-3' oligonucleotide was extended in a 20 μ l reaction mixture in the presence of 2 μ l of [α - 32 P]dTTP (10 mCi/ml, 400 Ci/mmol; 1 Ci=37 GBq), and 125 μ M dGTP as described (Lingner *et al.*, Genes Develop., 8:1984 [1994]) and loaded onto an 8% PAGE sequencing gel as described.

The results of this study are shown in Figure 28. In lane 1, there is no
10 telomerase present (*i.e.*, a negative control); lanes 2, 5, 8, and 11 contained 0.14 fmol telomerase; lanes 3, 6, 9, and 12 contained 0.42 fmol telomerase; and lanes 4, 7, 10, and 13 contained 1.3 fmol telomerase. Activity was quantitation using a PhosphorImager (Molecular Dynamics) using the manufacturer's instructions. It was determined that under these conditions, 1 fmol of affinity-purified telomerase incorporated 21 fmol of
15 dTTP in 30 minutes.

As shown in Figure 28, the specific activity of the telomerase did not change significantly through the purification procedure. Affinity-purified telomerase was fully active. However, it was determined that at high concentrations, an inhibitory activity was detected and the activity of crude extracts was not linear. Thus, in the
20 assay shown in Figure 28, the crude extract was diluted 700-7000-fold. Upon purification, this inhibitory activity was removed and no inhibitory effect was detected in the purified telomerase preparations, even at high enzyme concentrations.

G. Gel Electrophoresis and Northern Blots

25 As stated in Part E, at each step in the purification of telomerase, the preparation was analyzed by three separate assays. This Example describes the gel electrophoresis and blotting procedures used to quantify telomerase RNA present in fractions and analyze the integrity of the telomerase ribonucleoprotein particle.

i) Denaturing Gels and Northern Blots

30 In this Example, synthetic T7-transcribed telomerase RNA of known concentration served as the standard. Throughout this investigation, the RNA

component was used as a measure of telomerase.

A construct for phage T7 RNA polymerase transcription of *E. aediculatus* telomerase RNA was produced, using (PCR). The telomerase RNA gene was amplified with primers that annealed to either end of the gene. The primer that annealed at the 5' end also encoded a hammerhead ribozyme sequence to generate the natural 5' end upon cleavage of the transcribed RNA, a T7-promoter sequence, and an *EcoRI* site for subcloning. The sequence of this 5' primer was 5'-GCGGGAATTCTAA TACGACTCACTATAGGGAAGAACTCTGATGAGGCCGAAAGGCCGAAACT CCACGAAAGTGGAGTAAGTTTCTCGATAATTGATCTGTAG-3'. The 3' primer included an *EarI* site for termination of transcription at the natural 3' end, and a *BamHI* site for cloning. The sequence of this 3' primer was 5'-CGGGGATCCTCTTCAAAAG ATGAGAGGACAGCAAAC-3'. The PCR amplification product was cleaved with *EcoRI* and *BamHI*, and subcloned into the respective sites of pUC19 (NEB), to give "pEaT7." The correctness of this insert was confirmed by DNA sequencing. T7 transcription was performed as described by Zaug *et al.*, Biochemistry 33:14935 [1994], with *EarI*-linearized plasmid. RNA was gel-purified and the concentration was determined (an A_{260} of 1 = 40 $\mu\text{g/ml}$). This RNA was used as a standard to determine the telomerase RNA present in various preparations of telomerase.

The signal of hybridization was proportional to the amount of telomerase RNA, and the derived RNA concentrations were consistent with, but slightly higher than those obtained by native gel electrophoresis. Comparison of the amount of whole telomerase RNA in whole cell RNA to serial dilutions of known T7 RNA transcript concentrations indicated that each *E. aediculatus* cell contained approximately 300,000 telomerase molecules.

Visualization of the telomerase was accomplished by Northern blot hybridization to its RNA component, using methods as described (Linger *et al.*, Genes Develop., 8:1984 [1994]). Briefly, RNA (less than or equal to 0.5 $\mu\text{g/lane}$) was resolved on an 8% PAGE and electroblotted onto a Hybond-N membrane (Amersham), as known in the art (see e.g., Sambrook *et al.*, 1989). The blot was hybridized overnight in 10 ml of 4x SSC, 10x Denhardt's solution, 0.1% SDS, and 50 $\mu\text{g/ml}$ denatured herring sperm DNA. After pre-hybridizing for 3 hours, 2×10^6 cpm

probe/ml hybridization solution was added. The randomly labelled probe was a PCR-product that covered the entire telomerase RNA gene. The blot was washed with several buffer changes for 30 minutes in 2x SSC, 0.1% SDS, and then washed for 1 hour in 0.1x SSC and 0.1% SDS at 45°C.

5

ii) Native Gels and Northern Blots

In this experiment, the purified telomerase preparation was run on native (*i.e.*, non-denaturing) gels of 3.5% polyacrylamide and 0.33% agarose, as known in the art and described (Lamond and Sproat, [1994], *supra*). The telomerase comigrated approximately with the xylene cyanol dye.

10

The native gel results indicated that telomerase was maintained as an RNP throughout the purification protocol. Figure 27 is a photograph of a Northern blot showing the mobility of the telomerase in different fractions on a non-denaturing gel as well as *in vitro* transcribed telomerase. In this figure, lane 1 contained 1.5 fmol telomerase RNA. lane 2 contained 4.6 fmol telomerase RNA. lane 3 contained 14 fmol telomerase RNA. lane 4 contained 41 fmol telomerase RNA. lane 5 contained nuclear extract (42 fmol telomerase). lane 6 contained Affi-Gel-heparin-purified telomerase (47 fmol telomerase). lane 7 contained affinity-purified telomerase (68 fmol), and lane 8 contained glycerol gradient-purified telomerase (35 fmol).

15

As shown in Figure 27, in nuclear extracts, the telomerase was assembled into an RNP particle that migrated slower than unassembled telomerase RNA. Less than 1% free RNA was detected by this method. However, a slower migrating telomerase RNP complex was also sometimes detected in extracts. Upon purification on the Affi-Gel-heparin column, the telomerase RNP particle did not change in mobility (Figure 27, lane 6). However, upon affinity purification the mobility of the RNA particle slightly increased (Figure 27, lane 7), perhaps indicating that a protein subunit or fragment had been lost. On glycerol gradients, the affinity-purified telomerase did not change in size, but approximately 2% free telomerase RNA was detectable (Figure 27, lane 8), suggesting that a small amount of disassembly of the RNP particle had occurred.

20

25

30

H. Telomerase Protein Composition

In this Example, the analysis of the purified telomerase protein composition are described.

Glycerol gradient fractions obtained as described in Part D, were separated on a 4-20% polyacrylamide gel (Novex). Following electrophoresis, the gel was stained with Coomassie brilliant blue. Figure 29 shows a photograph of the gel. Lanes 1 and 2 contained molecular mass markers (Pharmacia) as indicated on the left side of the gel shown in Figure 29. Lanes 3-5 contained glycerol gradient fraction pools as indicated on the top of the gel (*i.e.*, lane 3 contained fractions 9-14, lane 4 contained fractions 15-22, and lane 5 contained fractions 23-32). Lane 4 contained the pool with 1 pmol of telomerase RNA. In lanes 6-9 BSA standards were run at concentrations indicated at the top of the gel in Figure 29 (*i.e.*, lane 6 contained 0.5 pmol BSA, lane 7 contained 1.5 pmol BSA, lane 8 contained 4.5 BSA, and lane 9 contained 15 pmol BSA).

As shown in Figure 29, polypeptides with molecular masses of 120 and 43 kDa co-purified with the telomerase. The 43 kDa polypeptide was observed as a doublet. It was noted that the polypeptide of approximately 43 kDa in lane 3 migrated differently than the doublet in lane 4; it may be an unrelated protein. The 120 kDa and 43 kDa doublet each stained with Coomassie brilliant blue at approximately the level of 1 pmol, when compared with BSA standards. Because this fraction contained 1 pmol of telomerase RNA, all of which was assembled into an RNP particle (See Figure 27, lane 8), there appear to be two polypeptide subunits that are stoichiometric with the telomerase RNA. However, it is also possible that the two proteins around 43 kDa are separate enzyme subunits.

Affinity-purified telomerase that was not subjected to fractionation on a glycerol gradient contained additional polypeptides with apparent molecular masses of 35 and 37 kDa, respectively. This latter fraction was estimated to be at least 50% pure. However, the 35 kDa and 37 kDa polypeptides that were present in the affinity-purified material were not reproducibly separated by glycerol gradient centrifugation. These polypeptides may be contaminants, as they were not visible in all activity-containing preparations.

I. Sedimentation Coefficient

The sedimentation coefficient for telomerase was determined by glycerol gradient centrifugation. In this Example, nuclear extract and affinity-purified telomerase were fractionated on 15-40% glycerol gradients containing 20 mM Tris-acetate, with 1 mM $MgCl_2$, 0.1 mM EDTA, 300 mM K₂Glu, and 1 mM DTT, at pH 7.5. Glycerol gradients were poured in 5 ml (13 x 51 mm) tubes, and centrifuged using an SW55Ti rotor (Beckman) at 55,000 rpm for 14 hours at 4°C.

Marker proteins were run in a parallel gradient and had a sedimentation coefficient of 7.6 S for alcohol dehydrogenase (ADH), 11.3 S for catalase, 17.3 S for apoferritin, and 19.3 S for thyroglobulin. The telomerase peak was identified by native gel electrophoresis of gradient fractions followed by blot hybridization to its RNA component.

Figure 30 is a graph showing the sedimentation coefficient for telomerase. As shown in this Figure, affinity-purified telomerase co-sedimented with catalase at 11.5 S, while telomerase in nuclear extracts sedimented slightly faster, peaking around 12.5 S. Therefore, consistent with the mobility of the enzyme in native gels, purified telomerase appears to have lost a proteolytic fragment or a loosely associated subunit.

The calculated molecular mass for telomerase, if it is assumed to consist of one 120 kDa protein subunit, one 43 kDa subunit, and one RNA subunit of 66 kDa, adds up to a total of 229 kDa. This is in close agreement with the 232 kDa molecular mass of catalase. However, the sedimentation coefficient is a function of the molecular mass, as well as the partial specific volume and the frictional coefficient of the molecule, both of which are unknown for the Euplotes telomerase RNP.

J. Substrate Utilization

In this Example, the substrate requirements of Euplotes telomerase were investigated. One simple model for DNA end replication predicts that after semi-conservative DNA replication, telomerase extends double-stranded, blunt-ended DNA molecules. In a variation of this model, a single-stranded 3' end is created by a helicase or nuclease after replication. This 3' end is then used by telomerase for binding and

extension.

To determine whether telomerase is capable of elongating blunt-ended molecules, model hairpins were synthesized with telomeric repeats positioned at their 3' ends. These primer substrates were gel-purified, 5'-end labelled with polynucleotide kinase, heated at 0.4 μ M to 80°C for 5 minutes, and then slowly cooled to room temperature in a heating block, to allow renaturation and helix formation of the hairpins. Substrate mobility on a non-denaturing gel indicated that very efficient hairpin formation was present, as compared to dimerization.

Assays were performed with unlabelled 125 μ M dGTP, 125 μ M dTTP, and 0.02 μ M 5'-end-labelled primer (5'-³²P-labelled oligonucleotide substrate) in 10 μ l reaction mixtures that contained 20 mM Tris-acetate, with 10 mM MgCl₂, 50 mM KCl, and 1 mM DTT, at pH 7.5. These mixtures were incubated at 25°C for 30 minutes. Reactions were stopped by adding formamide loading buffer (*i.e.*, TBE, formamide, bromthymol blue, and cyanol, Sambrook, 1989, *supra*).

Primers were incubated without telomerase (" -"), with 5.9 fmol of affinity-purified telomerase (" -"), or with 17.6 fmol of affinity-purified telomerase (" - - -"). Affinity-purified telomerase used in this assay was dialyzed with a membrane having a molecular cut-off of 100 kDa, in order to remove the displacement oligonucleotide. Reaction products were separated on an 8% PAGE/urea gel containing 36% formamide, to denature the hairpins. The sequences of the primers used in this study, as well as their lane assignments are shown in Table 6.

TABLE 6. Primer Sequences

| Lane | Primer Sequence (5' to 3') |
|-------|--|
| 1-3 | C ₄ (A ₄ C ₄) ₃ CACA(G ₄ T ₄) ₃ G ₄ |
| 4-6 | C ₂ (A ₄ C ₄) ₃ CACA(G ₄ T ₄) ₃ G ₄ |
| 7-9 | (A ₄ C ₄) ₃ CACA(G ₄ T ₄) ₃ G ₄ |
| 10-12 | A ₂ C ₄ (A ₄ C ₄) ₂ CACA(G ₄ T ₄) ₃ G ₄ |
| 13-15 | C ₄ (A ₄ C ₄) ₂ CACA(G ₄ T ₄) ₃ |
| 16-18 | (A ₄ C ₄) ₃ CACA(G ₄ T ₄) ₃ |
| 19-21 | A ₂ C ₄ (A ₄ C ₄) ₂ CACA(G ₄ T ₄) ₃ |
| 22-24 | C ₄ (A ₄ C ₄) ₂ CACA(G ₄ T ₄) ₃ |
| 25-27 | C ₂ (A ₄ C ₄) ₃ CACA(G ₄ T ₄) ₃ |
| 28-30 | (A ₄ C ₄) ₂ CACA(G ₄ T ₄) ₃ |

The gel results are shown in Figure 31. Lanes 1-15 contained substrates with telomeric repeats ending with four G residues. Lanes 16-30 contained substrates with telomeric repeats ending with four T residues. The putative alignment on the telomerase RNA template is indicated in Figure 32. It was assumed that the primer sets anneal at two very different positions in the template shown in Figure 32 (*i.e.*, Panel A and Panel B, respectively). This may have affected their binding and/or elongation rate.

Figure 33 shows a lighter exposure of lanes 25-30 in Figure 31. The lighter exposure of Figure 33 was taken to permit visualization of the nucleotides that are added and the positions of pausing in elongated products. Percent of substrate elongated for the third lane in each set was quantified on a PhosphorImager, as indicated on the bottom of Figure 31.

The substrate efficiencies for these hairpins were compared with double-stranded telomere-like substrates with overhangs of differing lengths. A model substrate that ended with four G residues (see lanes 1-15 of Figure 31) was not elongated when it was blunt ended (see lanes 1-3). However, slight extension was observed with an overhang length of two bases; elongation became efficient when the overhang was at least 4 bases in length. The telomerase acted in a similar manner with

a double-stranded substrate that ended with four T residues, with a 6-base overhang required for highly efficient elongation. In Figure 31, the faint bands below the primers in lanes 10-15 that are independent of telomerase represent shorter oligonucleotides in the primer preparations.

5 The lighter exposure of lanes 25-30 in Figure 33 shows a ladder of elongated products, with the darkest bands correlating with the putative 5' boundary of the template (as described by Lingner *et al.*, Genes Develop., 8:1984 [1994]). The abundance of products that correspond to other positions in the template suggested that pausing and/or dissociation occurs at sites other than the site of translocation with the
10 purified telomerase.

As shown in Figure 31, double-stranded, blunt-ended oligonucleotides were not substrates for telomerase. To determine whether these molecules would bind to telomerase, a competition experiment was performed. In this experiment, 2 nM of 5'-end labeled substrate with the sequence (G₄T₄), or a hairpin substrate with a six base
15 overhang were extended with 0.125 nM telomerase (Figure 31, lanes 25-27). Although the same unlabeled oligonucleotide substrates competed efficiently with labeled substrate for extension, no reduction of activity was observed when the double-stranded blunt-ended hairpin oligonucleotides were used as competitors, even in the presence of 100-fold excess hairpins.

20 These results indicated that double-stranded, blunt-ended oligonucleotides cannot bind to telomerase at the concentrations and conditions tested in this Example. Rather, a single-stranded 3' end is required for binding. It is likely that this 3' end is required to base pair with the telomerase RNA template.

25 K. Cloning & Sequencing of the 123 kDa Polypeptide

In this Example, the cloning of the 123 kDa polypeptide of Euplotes telomerase (*i.e.*, the 123 kDa protein subunit) is described. In this study, an internal fragment of the telomerase gene was amplified by PCR, with oligonucleotide primers designed to match peptide sequences that were obtained from the purified polypeptide
30 obtained in Part D, above. The polypeptide sequence was determined using the nanoES tandem mass spectroscopy methods known in the art and described by Calvio

et al., RNA 1:724-733 [1995]. The oligonucleotide primers used in this Example had the following sequences, with positions that were degenerate shown in parentheses--5'-TCT(G/A)

AA(G/A)TA(G/A)TG(T/G/A)GT(G/A/T/C)A(T/G/A)(G/A)TT(G/A)TTCAT-3', and

5'-GCGGATCCATGAA(T/C)CC(A/T)GA(G/A)AA(T/C)CC(A/T)AA(T/C)GT-3'.

A 50 µl reaction contained 0.2 mM dNTPs, 0.15 µg *E. aediculatus* chromosomal DNA, 0.5 µl *Taq* (Boehringer-Mannheim), 0.8 µg of each primer, and 1x reaction buffer (Boehringer-Mannheim). The reaction was incubated in a thermocycler (Perkin-Elmer), using the following--5 minutes at 95°C, followed by 30 cycles of 1 minute at 94°C, 1 minute at 52°C, and 2 minutes at 72°C. The reaction was completed by a 10 minute incubation at 72°C.

A genomic DNA library was prepared from the chromosomal *E. aediculatus* DNA by cloning blunt-ended DNA into the *Sma*I site of pCR-Script plasmid vector Figure 14(Stratagene). This library was screened by colony hybridization, with the radiolabelled, gel-purified PCR product. Plasmid DNA of positive clones was prepared and sequenced by the dideoxy method (Sanger *et al.*, Proc. Natl. Acad. Sci., 74:5463 [1977]) or manually, through use of an automated sequencer (ABI). The DNA sequence of the gene encoding this polypeptide is shown in Figure 13. The start codon in this sequence inferred from the DNA sequence, is located at nucleotide position 101, and the open reading frame ends at position 3193. The genetic code of *Euplotes* differs from other organisms in that the "UGA" codon encodes a cysteine residue. The amino acid sequence of the polypeptide inferred from the DNA sequence is shown in Figure 14, and assumes that no unusual amino acids are inserted during translation and no post-translational modification occurs.

L. Cloning & Sequencing of the 43 kDa Polypeptide

In this Example, the cloning of the 43 kDa polypeptide of telomerase (*i.e.*, the 43 kDa protein subunit) is described. In this study, an internal fragment of the corresponding telomerase gene was amplified by PCR, with oligonucleotide primers designed to match peptide sequences that were obtained from the purified polypeptide obtained in Part D, above. The polypeptide sequence was determined using the nanoES

tandem mass spectroscopy methods known in the art and described by Calvio *et al.*,
supra. The oligonucleotide primers used in this Example had the following sequences—
5'-NNNGTNAC(C/T/A)GG(C/T/A)AT(C/T/A)AA(C/T)AA-3', and 5'-(T/G/A)GC
(T/G/A)GT(C/T)TC(T/C)TG(G/A)TC(G/A)TT(G/A)TA-3'. In this sequence, "N"

5 indicates the presence of any of the four nucleotides (*i.e.*, A, T, G, or C).

The PCR was performed as described in Part K.

A genomic DNA library was prepared and screened as described in Part
K. The DNA sequence of the gene encoding this polypeptide is shown in Figure 34.
Three potential reading frames are shown for this sequence, as shown in Figure 35.
10 For clarity, the amino acid sequence is indicated below the nucleotide sequence in all
three reading frames. These reading frames are designated as "a," "b," and "c". A
possible start codon is encoded at nucleotide position 84 in reading frame "c." The
coding region could end at position 1501 in reading frame "b." Early stop codons,
indicated by asterisks in this figure, occur in all three reading frames between
15 nucleotide position 337-350.

The "La-domain" is indicated in bold-face type. Further downstream,
the protein sequence appears to be encoded by different reading frames, as none of the
three frames is uninterrupted by stop codons. Furthermore, peptide sequences from
purified protein are encoded in all three frames. Therefore, this gene appears to contain
20 intervening sequences, or in the alternative, the RNA is edited. Other possibilities
include ribosomal frame-shifting or sequence errors. However, the homology to the
La-protein sequence remains of significant interest. Again, in *Euplotes*, the "UGA"
codon encodes a cysteine residue.

25 M. Amino Acid and Nucleic Acid Comparisons

In this Example, comparisons between various reported sequences and
the sequences of the 123 kDa and 43 kDa telomerase subunit polypeptides were made.

i) Comparisons with the 123 kDa *E. aediculatus* Telomerase Subunit

30 The amino acid sequence of the 123 kDa *Euplotes aediculatus*
polypeptide was compared with the sequence of the 80 kDa telomerase protein subunit

of *Tetrahymena thermophila* (GenBank accession #U25641) to investigate their similarity. The nucleotide sequence as obtained from GenBank encoding this protein is shown in Figure 42. The amino acid sequence of this protein as obtained from GenBank is shown in Figure 43. The sequence comparison between the 123 kDa *E. aediculatus* and 80 kDa *T. thermophila* is shown in Figure 36. In this figure, the *E. aediculatus* sequence is the upper sequence, while the *T. thermophila* sequence is the lower sequence. The observed identity was determined to be approximately 19%, while the percent similarity was approximately 45%, values similar to what would be observed with any random protein sequence. In Figures 36-39, identities are indicated by vertical bars, while single dots between the sequences indicate somewhat similar amino acids, and double dots between the sequences indicate more similar amino acids.

The amino acid sequence of the 123 kDa *Euplotes aediculatus* polypeptide was also compared with the sequence of the 95 kDa telomerase protein subunit of *Tetrahymena thermophila* (GenBank accession #U25642), to investigate their similarity. The nucleotide sequence as obtained from GenBank encoding this protein is shown in Figure 44. The amino acid sequence of this protein as obtained from GenBank is shown in Figure 45. This sequence comparison is shown in Figure 37. In this figure, the *E. aediculatus* sequence is the upper sequence, while the *T. thermophila* sequence is the lower sequence. The observed identity was determined to be approximately 20%, while the percent similarity was approximately 43%, values similar to what would be observed with any random protein sequence.

Significantly, the amino acid sequence of the 123 kDa *E. aediculatus* polypeptide contains the five motifs characteristic of reverse transcriptases. The 123 kDa polypeptide was also compared with the polymerase domains of various reverse transcriptases. Figure 40 shows the alignment of the 123 kDa polypeptide with the putative yeast homolog (L8543.12 or ESTp). The amino acid sequence of L8543.12 obtained from GenBank is shown in Figure 46.

Four motifs (A, B, C, and D) were included in this comparison. In this Figure 40, highly conserved residues are indicated by white letters on a black background. Residues of the *E. aediculatus* sequences that are conserved in the other

sequence are indicated in bold; the "h" indicates the presence of a hydrophobic amino acid. The numerals located between amino acid residues of the motifs indicates the length of gaps in the sequences. For example, the "100" shown between motifs A and B reflects a 100 amino acid gap in the sequence between the motifs.

5 As noted above, Genbank searches identified a yeast protein (Genbank accession #u20618), and gene L8543.12 (Est2) containing or encoding amino acid sequence that shows some homology to the *E. aediculatus* 123 kDa telomerase subunit. Based on the observations that both proteins contain reverse transcriptase motifs in their C-terminal regions; both proteins share similarity in regions outside the reverse
10 transcriptase motif; the proteins are similarly basic (pI = 10.1 for *E. aediculatus* and pI=10.0 for the yeast); and both proteins are large (123 kDa for *E. aediculatus* and 103 kDa for the yeast), these sequences comprise the catalytic core of their respective telomerases. It was contemplated based on this observation of homology in two phylogenetically distinct organisms as *E. aediculatus* and yeast, that human telomerase
15 would contain a protein that has the same characteristics (*i.e.*, reverse transcriptase motifs, is basic, and large (> 100 kDa)).

ii) Comparisons with the 43 kDa *E. aediculatus* Telomerase Subunit

The amino acid sequence of the "La-domain" of the 43 kDa *Euplotes*
20 *aediculatus* polypeptide was compared with the sequence of the 95 kDa telomerase protein subunit of *Tetrahymena thermophila* (described above) to investigate their similarity. This sequence comparison is shown in Figure 38, while the *T. thermophila* sequence is the lower sequence. The observed identity was determined to be approximately 23%, while the percent similarity was approximately 46%, values
25 similar to what would be observed with any random protein sequence.

The amino acid sequence of the "La-domain" of the 43 kDa *Euplotes*
aediculatus polypeptide was compared with the sequence of the 80 kDa telomerase protein subunit of *Tetrahymena thermophila* (described above) to investigate their similarity. This sequence comparison is shown in Figure 39. In this figure, the *E.*
30 *aediculatus* sequence is the upper sequence, while the *T. thermophila* sequence is the lower sequence. The observed identity was determined to be approximately 26%, while

the percent similarity was approximately 49%, values similar to what would be observed with any random protein sequence.

The amino acid sequence of a domain of the 43 kDa *E. aediculatus* polypeptide was also compared with La proteins from various other organisms. These comparisons are shown in Figure 41. In this Figure, highly conserved residues are indicated by white letters on a black background. Residues of the *E. aediculatus* sequences that are conserved in the other sequence are indicated in bold.

N. Identification of Telomerase Protein Subunits in Another Organism

In this Example, the sequences identified in the previous Examples above were used to identify the telomerase protein subunits of *Oxytricha trifallax*, a ciliate that is very distantly related to *E. aediculatus*. Primers were chosen based on the conserved region of the *E. aediculatus* 123 kDa polypeptide which comprised the reverse transcriptase domain motifs. Suitable primers were synthesized and used in a PCR reaction with total DNA from *Oxytricha*. The *Oxytricha* DNA was prepared according to methods known in the art. The PCR products were then cloned and sequenced using methods known in the art.

The oligonucleotide sequences used as the primers were as follows:

5'-(T/C)A(A/G)AC(T/A/C)AA(G/A)GG(T/A/C)AT(T/C)CC(C/T/A)(C/T)A(G/A)GG-3' and 5'-(G/A/T)GT(G/A/T)ATNA(G/A)NA(G/A)(G/A)TA(G/A)TC(G/A)TC-3'. Positions that were degenerate are shown in parentheses, with the alternative bases shown within the parenthesis. "N" represents any of the four nucleotides.

In the PCR reaction, a 50 µl reaction contained 0.2 mM dNTPs, 0.3 µg *Oxytricha trifallax* chromosomal DNA, 1 µl *Taq* polymerase (Boehringer-Mannheim), 2 micromolar of each primer, 1x reaction buffer (Boehringer-Mannheim). The reaction was incubated in a thermocycler (Perkin-Elmer) under the following conditions: 5 min at 95°C, 30 cycles consisting of 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C, followed by a 10 min incubation at 72°C. The PCR-product was gel-purified and sequenced by the dideoxy-method (e.g., Sanger *et al.*, Proc. Natl. Acad. Sci. 74, 5463-5467 (1977)).

The deduced amino acid sequence of the PCR product was determined

and compared with the *E. aediculatus* sequence. Figure 47 shows the alignment of these sequences, with the *O. trifallax* sequence shown in the top row, and the *E. aediculatus* sequence shown in the bottom row. As can be seen from this figure, there is a great deal of homology between the *O. trifallax* polypeptide sequence identified in this Example with the *E. aediculatus* polypeptide sequence. Thus, it is clear that the sequences identified in the present invention are useful for the identification of homologous telomerase protein subunits in other eukaryotic organisms. Indeed, development of the present invention has identified homologous telomerase sequences in multiple, diverse species, as described herein.

O. Identification of *Tetrahymena* Telomerase Sequences

In this Example, a *Tetrahymena* clone was produced that shares homology with the *Euplotes* sequences, and EST2p.

This experiment utilized PCR with degenerate oligonucleotide primers directed against conserved motifs to identify regions of homology between *Tetrahymena*, *Euplotes*, and EST2p sequences. The PCR method used in this Example is a novel method designed to amplify specifically rare DNA sequences from complex mixtures. This method avoids the problem of amplification of DNA products with the same PCR primer at both ends (i.e., single primer products) commonly encountered in PCR cloning methods. These single primer products produce unwanted background and can often obscure the amplification and detection of the desired two-primer product. The method used in this experiment preferentially selects for two-primer products. In particular, one primer is biotinylated and the other is not. After several rounds of PCR amplification, the products are purified using streptavidin magnetic beads and two primer products are specifically eluted using heat denaturation. This method finds use in settings other than the experiments described in this Example. Indeed, this method finds use in application in which it is desired to specifically amplify rare DNA sequences, including the preliminary steps in cloning methods such as 5' and 3' RACE, and any method that uses degenerate primers in PCR.

A first PCR run was conducted using *Tetrahymena* template macronuclear DNA isolated using methods known in the art. and the 24-mer forward

primer with the sequence 5' biotin-GCCTATTT(TC)TT(TC)TA(TC)(GATC)(GATC)
(GATC)AC(GATC)GA-3' designated as "K231," corresponding to the FFYXTE
region, and the 23-mer reverse primer with the sequence 5'- CCAGATAT(GATC)A
(TGA)(GATC)A(AG)(AG)AA(AG)TC(AG)TC- 3', designated as "K220,"

5 corresponding to the DDFL(FIL)I region. This PCR reaction contained 2.5 µl DNA
(50 ng), 4 µl of each primer (20 µM), 3 µl 10x PCR buffer, 3 µl 10x dNTPs, 2 µl Mg,
0.3 µl *Taq*, and 11.2 µl dH₂O. The mixture was cycled for 8 cycles of 94°C for 45
seconds, 37°C for 45 seconds, and 72 °C for 1 minute.

This PCR reaction was bound to 200 µl streptavidin magnetic beads,
10 washed with 200 µl TE, resuspended in 20 µl dH₂O and then heat-denatured by boiling
at 100°C for 2 minutes. The beads were pulled down and the eluate removed. Then,
2.5 µl of this eluate was subsequently reamplified using the above conditions, with the
exception being that 0.3 µl of α -³²P dATP was included, and the PCR was carried out
for 33 cycles. This reaction was run a 5% denaturing polyacrylamide gel, and the
15 appropriate region was cut out of the gel. These products were then reamplified for an
additional 34 cycles, under the conditions listed above, with the exception being that a
42°C annealing temperature was used.

A second PCR run was conducted using *Tetrahymena* macronuclear
DNA template isolated using methods known in the art, and the 23-mer forward primer
20 with the sequence 5'- ACAATG(CA)G(GATC)(TCA)T(GATC)(TCA)T(GATC)CC
(GATC)AA(AG)AA-3' . designated as "K228," corresponding to the region
R(LI)(LI)PKK , and a reverse primer with the sequence 5'-ACGAATC(GT)(GATC)GG
(TAG)AT(GATC)(GC)(TA)(AG)TC(AG)TA(AG)CA 3' , designated "K224,"
corresponding to the CYDSIPR region. This PCR reaction contained 2.5 µl DNA (50
25 ng), 4 µl of each primer (20 µM), 3 µl 10x PCR buffer, 3 µl 10x dNTPs, 2 µl Mg, 0.3
µl α -³²P dATP, 0.3 µl *Taq*, and 10.9 µl dH₂O. This reaction was run on a 5%
denaturing polyacrylamide gel, and the appropriate region was cut out of the gel. These
products were reamplified for an additional 34 cycles, under the conditions listed
above, with the exception being that a 42°C annealing temperature was used.

30 Ten µl of the reaction product from run 1 were bound to streptavidin-
coated magnetic beads in 200 µl TE. The beads were washed with 200 µl TE, and then

resuspended in 20 µl of dH₂O, heat denatured, and the eluate was removed. The reaction product from run 2 was then added to the beads and diluted with 30 µl 0.5x SSC. The mixture was heated from 94°C to 50°C. The eluate was removed and the beads were washed three times in 0.5x SSC at 55°C. The beads were then resuspended
5 in 20 µl dH₂O, heat denatured, and the eluate was removed, designated as "round 1 eluate" and saved.

To isolate the *Tetrahymena* band, the round 1 eluate was reamplified with the forward primer K228 and reverse primer K227 with the sequence
5'- CAATTCTC(AG)TA(AG)CA(GATC)(CG)(TA)(CT)TT(AGT)AT(GA)TC-3' ,
10 corresponding to the DIKSCYD region. The PCR reactions were conducted as described above. The reaction products were run on a 5% polyacrylamide gel; the band corresponding to approximately 295 nucleotides was cut from the gel and sequenced.

The clone designated as 168-3 was sequenced. The DNA sequence (including the primer sequences) was found to be:
15 GATTACTCCCGAAGAAAGGATCTTTCCGTCCAATCATGACTTTCTTAAGAAA
GGACAAGCAAAAAAATATTAAGTTAAATCTAAATTAAATTCTAATGGATAG
CCAACTTGTGTTTAGGAATTTAAAAGACATGCTGGGATAAAAGATAGGATA
CTCAGTCTTTGATAATAAACAAATTTTCAGAAAAATTTGCCTAATTCATAGAG
AAATGGAAAAATAAAGGAAGACCTCAGCTATATTATGTCACTCTAGACATA
20 AAGACTTGCTAC.

Additional sequence of this gene was obtained by PCR using one unique primer designed to match the sequence from 168-3 ("K297" with the sequence
5'-GAGTGACATAATATACGTGA-3'; and the K231 (FFYXTE) primer. The sequence of the fragment obtained from this reaction, together with 168-3 is as follows
25 (without the primer sequences):

AAACACAAGGAAGGAAGTCAAATATTCTATTACCGTAAACCAATATGGAA
ATTAGTGAGTAAATTAATCTATTGTCAAAGTAAGAATTTAGTTTTCTGAAAAG
AATAAATAAATGAAAAATAATTTTATCAAAAAATTTAGCTTGAAGAGGAG
AATTTGGAAAAAGTTGAAGAAAAATTGATACCAGAAGATTCATTTTAGAAA
30 TACCCTCAAGGAAAGCTAAGGATTATACCTAAAAAAGGATCTTTCCGTCCA
ATCATGACTTTCTTAAGAAAGGACAAGCAAAAAAATATTAAGTTAAATCTA

AATTAAATTCTAATGGATAGCCAACTTGTGTTTAGGAATTTAAAAGACATG
 CTGGGATAAAAGATAGGATACTCAGTCTTTGATAATAAACAAATTCAGAA
 AAATTTGCCTAATTCATAGAGAAATGGAAAAATAAAGGAAGACCTCAGCTA
 TATTATGTCACTCTA.

5 The amino acid sequence corresponding to this DNA fragment was
 found to be:
 KHKEGSQIFYRKPIWKLVSCLTIVKVRIQFSEKNKQMKNFYQKIQLEENLE
 KVEEKLIPEDSFQKYPQGKLRIIPKKGSRPIMTFLRKDKQKNIKLNLNQILMDS
 QLVFRNLKDMLGQKIGYSVFDNKQISEKFAQFIEKWKNKGRPQLYYVTL.

10 This amino acid sequence was then aligned with other telomerase genes
 (EST2p, and *Euplotes*). The alignment is shown in Figure 53. A consensus sequence is
 also shown in this Figure.

P. Identification of *Schizosaccharomyces pombe* Telomerase Sequences

15 In this Example, the *tez1* sequence of *S. pombe* was identified as a
 homolog of the *E. aediculatus* p123, and *S. cerevisiae* Est2p.

Figure 55 provides an overall summary of these experiments. In this
 Figure, the top portion (Panel A) shows the relationship of two overlapping genomic
 clones, and the 5825 bp portion that was sequenced. The region designated at "*tez1*" is
 20 the protein coding region, with the flanking sequences indicated as well, the box
 underneath the 5825 bp region is an approximately 2 kb *HindIII* fragment that was used
 to make the *tez1* disruption construct, as described below.

The bottom half of Figure 55 (Panel B) is a "close-up" schematic of this
 same region of DNA. The sequence designated as "original PCR" is the original
 25 degenerate PCR fragment that was generated with a degenerate oligonucleotide primer
 pair designed based on *Euplotes* sequence motif 4 (B') and motif 5 (C), as described.

i) PCR With Degenerate Primers

30 PCR using degenerate primers was used to find the homolog of the *E.*
aediculatus p123 in *S. pombe*. Figure 56 shows the sequences of the degenerate
 primers (designated as "poly 4" and "poly 1") used in this reaction. The PCR runs were

conducted using the same buffer as described in previous Examples (See e.g., Part K, above), with a 5 minute ramp time at 94°C, followed by 30 cycles of 94°C for 30 seconds, 50°C for 45 seconds, and 72°C for 30 seconds, and 7 minutes at 72°C, followed by storage at 4°C. PCR runs were conducted using varied conditions, (i.e., various concentrations of *S. pombe* DNA and MgCl₂ concentrations). The PCR products were run on agarose gels and stained with ethidium bromide as described above. Several PCR runs resulted in the production of three bands (designated as "T," "M," and "B"). These bands were re-amplified and run on gels using the same conditions as described above. Four bands were observed following this re-amplification ("T," "M1," "M2," and "B"), as shown in Figure 57. These four bands were then re-amplified using the same conditions as described above. The third band from the top of the lane in Figure 57 was identified as containing the correct sequence for a telomerase protein. The PCR product designated as M2 was found to show a reasonable match with other telomerase proteins, as indicated in Figure 58. In addition to the alignment shown, this Figure also shows the actual sequence of *tez1*. In this Figure, the asterisks indicate residues shared with all four sequences (*Oxytricha* "Ot"; *E. adiculatus* "Ea_p123"; *S. cerevisiae* "Sc_p103"; and M2), while the circles (i.e., dots) indicate similar amino acid residues.

20 ii) 3' RT PCR

To obtain additional sequence information, 3' and 5' RT PCR were conducted on the telomerase candidate identified in Figure 58. Figure 59 provides a schematic of the 3' RT PCR strategy used. First, cDNA was prepared from mRNA using the oligonucleotide primer "Q_T," (5'-CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GCT TTT TTT TTT TTT TT-3'), then using this cDNA as a template for PCR with "Q_O" (5'-CCA GTG AGC AGA GTG ACG-3'), and a primer designed based on the original degenerated PCR reaction (i.e., "M2-T" with the sequence 5'-G TGT CAT TTC TAT ATG GAA GAT TTG ATT GAT G-3'). The second PCR reaction (i.e., nested PCR) with "Q_I" (5'-GAG GAC TCG AGC TCA AGC-3'), and another PCR primer designed with sequence derived from the original degenerate PCR reaction or "M2-T2" (5'-AC CTA TCG TTT ACG AAA AAG AAA

GGA TCA GTG-3'). The buffers used in this PCR were the same as described above, with amplification conducted beginning with a ramp up of 94° for 5 min, followed by 30 cycles of 94° for 30 sec, 55°C for 30 sec, and 72°C for 3 min, followed by 7 minutes at 72°C. The reaction products were stored at 4°C until use.

5

iii) Screening of Genomic and cDNA Libraries

After obtaining this additional sequence information, several genomic and cDNA libraries were screened to identify any libraries that contain this telomerase candidate gene. The approach used, as well as the libraries and results are shown in Figure 60. In this Figure, Panel A lists the libraries tested in this experiment; Panel B shows the regions used; Panels C and D show the dot blot hybridization results obtained with these libraries. Positive libraries were then screened by colony hybridization to obtain genomic and cDNA version of *tez1* gene. In this experiment, approximately 3×10^4 colonies from the *HindIII* genomic library were screened and six positive clones were identified (approximately 0.01%). DNA was then prepared from two independent clones (A5 and B2). Figure 61 shows the results obtained with the *HindIII*-digested A5 and B2 positive genomic clones.

In addition, cDNA REP libraries were used. Approximately 3×10^5 colonies were screened, and 5 positive clones were identified (0.002%). DNA was prepared from three independent clones (2-3, 4-1, and 5-20). In later experiments, it was determined that clones 2-3 and 5-20 contained identical inserts.

20

iv) 5' RT PCR

As the cDNA version of gene produced to this point was not complete, 5' RT-PCR was conducted to obtain a full length clone. The strategy is schematically shown in Figure 62. In this experiment, cDNA was prepared using DNA oligonucleotide primer "M2-B" (5'-CAC TGA TCC TTT CTT TTT CGT AAA CGA TAG GT-3') and "M2-B2" (5'-C ATC AAT CAA ATC TTC CAT ATA GAA ATG ACA-3'), designed from known regions of *tez1* identified previously. An oligonucleotide linker PCR Adapt *SfiI* with a phosphorylated 5' end ("P") (P-GGG CCG TGT TGG CCT AGT TCT CTG CTC-3'; was then ligated at the 3' end of this

25

30

cDNA, and this construct was used as the template for nested PCR. In the first round of PCR, PCR Adapt SFI and M2-B were used as the primers; while PCR Adapt SfilI (5-GAG GAG GAG AAG AGC AGA GAA CTA GGC CAA CAC GCC CC-3'), and M2-B2 were used as primers in the second round. Nested PCR was used to increase
5 specificity of reaction.

v) Sequence Alignments

Once the sequence of *tez1* was identified, it was compared with sequences previously described. Figure 63 shows the alignment of RT domains from
10 telomerase catalytic subunits of *S. pombe* ("S.p. Tez1p"), *S. cerevisiae* ("S.c. Est2p"), and *E. aediculatus* p123 ("E.a. p123"). In this Figure, "h" indicates hydrophobic residues, while "p" indicates small polar residues, and "c" indicates charged residues. The amino acid residues indicated above the alignment show a known consensus RT motif of Y. Xiong and T.H. Eickbush (Y. Xiong and T.H. Eickbush, EMBO J., 9:
15 3353-3362 [1990]). The asterisks indicate the residues that are conserved for all three proteins. "Motif O" is identified herein and in Figure 63 as a motif specific to this telomerase subunit and not found in reverse transcriptases in general. It is therefore valuable in identifying other amino acid sequences as telomerase catalytic subunits.

Figure 64 shows the alignment of entire sequences from *Euplotes*
20 ("Ea_p123"), *S. cerevisiae* ("Sc_Est2p"), and *S. pombe* ("Sp_Tez1p"). In Panel A, the shaded areas indicate residues shared between two sequences. In Panel B, the shaded areas indicate residues shared between all three sequences.

vi) Genetic Disruption of *tez1*

25 In this Example, the effects of disruption of *tez1* were investigated. As telomerase is involved in telomere maintenance, it was hypothesized that if *tez1* were indeed a telomerase component, disruption of *tez1* would cause gradual telomere shortening.

In these experiments, homologous recombination was used to disrupt the
30 *tez1* gene in *S. pombe* specifically. This approach is schematically illustrated in Figure 65. As indicated in Figure 65, wild type *tez1* was replaced with a fragment containing

the *ura4* or *LEU2* marker.

The disruption of *tez1* gene was confirmed by PCR (Figure 66), and a Southern blot was performed to check for telomere length. Figure 67 shows the Southern blot results for this experiment. Because an *Apal* restriction enzyme site is present immediately adjacent to telomeric sequence in *S. pombe*, *Apal* digestion of *S. pombe* genomic DNA preparations permits analysis of telomere length. Thus, DNA from *S. pombe* was digested with *Apal* and the digestion products were run on an agarose gel and probed with a telomeric sequence-specific probe to determine whether the telomeres of disrupted *S. pombe* cells were shortened. The results are shown in Figure 67. From these results, it was clear that disruption of the *tez1* gene caused a shortening of the telomeres.

Q. Cloning and Characterization of Human Telomerase Protein and cDNA

In this Example, the nucleic and amino acid sequence information for human telomerase was determined. Partial homologous sequences were first identified in a BLAST search conducted using the *Euplotes* 123 kDa peptide and nucleic acid sequences, as well as *Schizosaccharomyces* protein and corresponding cDNA (*tez1*) sequences. The human sequences (also referred to as "hTCP1.1") were identified from a partial cDNA clone (clone 712562). Sequences from this clone were aligned with the sequences determined as described in previous Examples.

Figure 1 shows the sequence alignment of the *Euplotes* ("p123"), *Schizosaccharomyces* ("tez1"), Est2p (i.e., the *S. cerevisiae* protein encoded by the *Est2* nucleic acid sequence, and also referred to herein as "L8543.12"), and the human homolog identified in this comparison search. Figure 51 shows the amino acid sequence of *tez1*, while Figure 52 shows the DNA sequence of *tez1*. In Figure 52, the introns and other non-coding regions, are shown in lower case, while the exons (i.e., coding regions) are shown in upper case.

As shown in the Figures, there are regions that are highly conserved among these proteins. For example, as shown in Figure 1, there are regions of identity in "Motif 0," "Motif 1," "Motif 2," and "Motif 3." The identical amino acids are indicated with an asterisk (*), while the similar amino acid residues are indicated by a

circle (•). This indicates that there are regions within the telomerase motifs that are conserved among a wide variety of eukaryotes, ranging from yeast to ciliates to humans. It is contemplated that additional organisms will likewise contain such conserved regions of sequence. Figure 49 shows the partial amino acid sequence of the human telomerase motifs, while Figure 50 shows the corresponding DNA sequence.

Sanger dideoxy sequencing and other methods were used, as known in the art to obtain complete sequence information of clone 712562. Some of the primers used in the sequencing are shown in Table 7. These primers were designed to hybridize to the clone), based on sequence complementarity to either plasmid backbone sequence or the sequence of the human cDNA insert in the clone.

Table 7. Primers

| Primer | Sequence |
|---------|----------------------|
| TCP1.1 | GTGAAGGCACTGTTTCAGCG |
| TCP1.2 | GTGGATGATTCTTGTTGG |
| TCP1.3 | ATGCTCCTGCGTTTGGTGG |
| TCP1.4 | CTGGACACTCAGCCCTTGG |
| TCP1.5 | GGCAGGTGTGCTGGACACT |
| TCP1.6 | TTTGATGATGCTGGCGATG |
| TCP1.7 | GGGGCTCGTCTTCTACAGG |
| TCP1.8 | CAGCAGGAGGATCTTGTAG |
| TCP1.9 | TGACCCCAGGAGTGGCACG |
| TCP1.10 | TCAAGCTGACTCGACACCG |
| TCP1.11 | CGGCGTGACAGGGCTGC |
| TCP1.12 | GCTGAAGGCTGAGTGTCC |
| TCP1.13 | TAGTCCATGTTTACAATCG |

From these experiments, it was determined that the EcoRI-NotI insert of clone 712562 contains only a partial open reading frame for the human telomerase protein, although it may encode an active fragment of that protein. The open reading

frame in the clone encodes an approximately 63 kD protein. The sequence of the longest open reading frame identified is shown in Figure 68. The ORF begins at the ATG codon with the "met" indicated in the Figure. The poly A tail at the 3' end of the sequence is also shown. Figure 69 shows a tentative, preliminary alignment of
5 telomerase reverse transcriptase proteins from the human sequence (human Telomerase Core Protein 1, "Hs TCP1"), *E. aediculatus* p123 ("Ep p123), *S. pombe* tez1 ("Sp Tez1"), *S. cerevisiae* EST2 (Sc Est2"), and consensus sequence. In this Figure various motifs are indicated.

To obtain a full-length clone, probing of a cDNA library and 5'-RACE
10 were used to obtain clones encoding portions of the previously uncloned regions. In these experiments, RACE (Rapid Amplification of cDNA Ends; See e.g., M.A. Frohman, "RACE: Rapid Amplification of cDNA Ends," in Innis *et al.* (eds), *PCR Protocols: A Guide to Methods and Applications* [1990], pp. 28-38; and Frohman *et al.*, Proc. Natl. Acad. Sci., 85:8998-9002 [1988]) was used to generate material for
15 sequence analysis. Four such clones were generated and used to provide additional 5' sequence information (pFWRP5, 6, 19, and 20).

In addition, human cDNA libraries (inserted into lambda) were probed with the EcoRI-NotI fragment of the clone. One lambda clone, designated "lambda 25-1.1" (ATCC accession #209024), was identified as containing complementary
20 sequences. Figure 75 shows a restriction map of this lambda clone. The human cDNA insert from this clone was subcloned as an EcoRI restriction fragment into the EcoRI site of commercially available phagemid pBluescriptII SK+ (Stratagene), to create the plasmid "pGRN121," which was deposited with the ATCC (ATCC accession #209016). Preliminary results indicated that plasmid pGRN121 contains the entire
25 open reading frame (ORF) sequence encoding the human telomerase protein.

The cDNA insert of plasmid pGRN121 was sequenced using techniques known in the art. Figure 70 provides a restriction site and function map of plasmid pGRN121 identified based on this preliminary work. The results of this preliminary sequence analysis are shown in Figure 71. From this analysis, and as shown in Figure
30 70, a putative start site for the coding region was identified at approximately 50 nucleotides from the EcoRI site (located at position 707), and the location of the

telomerase-specific motifs, "FFYVTE", "PKP," "AYD," "QG", and "DD," were identified, in addition to a putative stop site at nucleotide #3571 (See, Figure 72, which shows the DNA and corresponding amino acid sequences for the open reading frames in the sequence ("a", "b", and "c"). However, due to the preliminary nature of the early sequencing work, the reading frames for the various motifs were found not to be in alignment.

Additional analysis conducted on the pGRN121 indicated that the plasmid contained significant portions from the 5'-end of the coding sequence not present on clone 712562. Furthermore, pGRN121 was found to contain a variant coding sequence that includes an insert of approximately 182 nucleotides. This insert was found to be absent from the clone. As with the *E. aediculatus* sequences, such variants can be tested in functional assays, such as telomerase assays to detect the presence of functional telomerase in a sample.

Further sequence analysis resolved the cDNA sequence of pGRN121 to provide a contiguous open reading frame that encodes a protein of molecular weight of approximately 127,000 daltons, and 1132 amino acids as shown in Figure 74. A refined map of pGRN121 based on this analysis, is provided in Figure 73. The results of additional sequence analysis of the hTERT cDNA are presented in Figure 16 (SEQUENCE ID NO: 1).

EXAMPLE 2

CORRELATION OF HTERT ABUNDANCE AND CELL IMMORTALITY

The relative abundance of hTERT mRNA was assessed in six telomerase-negative mortal cell strains and six telomerase-positive immortal cell lines (Figure 5).

The steady state level of hTERT mRNA was significantly increased in immortal cell lines that had previously been shown to have active telomerase. Lower levels of the hTERT mRNA were detected in some telomerase-negative cell strains.

RT-PCR for hTERT, hTR, TP1 (telomerase-associated protein related to *Tetrahymena* p80 [Harrington et al., 1997, *Science* 275:973; Nakayama et al., 1997, *Cell* 88:875]) and GAPDH (to normalize for equal amounts of RNA template) was carried out on RNA derived from the following cells: (1) human fetal lung fibroblasts

GFL, (2) human fetal skin fibroblasts GFS, (3) adult prostate stromal fibroblasts 31 YO, (4) human fetal knee synovial fibroblasts HSF, (5) neonatal foreskin fibroblasts BJ, (6) human fetal lung fibroblasts IMR90, and immortalized cell lines: (7) melanoma LOX IMVI, (8) leukemia U251, (9) NCI H23 lung carcinoma, (10) colon adenocarcinoma SW620, (11) breast tumor MCF7, (12) 293 adenovirus E1 transformed human embryonic kidney cell line.

hTERT nucleic acid was amplified from cDNA using oligonucleotide primers LT5 and LT6 (Table 2) for a total of 31 cycles (94°C 45s, 60°C 45s, 72°C 90s). GAPDH was amplified using primers K136 (5'-CTCAGACACCATGGGGAA GGTGA) and K137 (5'-ATGATCTTGAGGCTGTTGTCATA) for a total of 16 cycles (94°C 45 s, 55°C 45 s, 72°C 90 s). hTERT was amplified using primers F3b (5'-TCTAA CCCTAACTGAGAAGGGCGTAG) and R3c (5'-GTTTGCTCTAGAATGAACGGTG GAAG) for a total of 22 cycles (94 °C 45s, 55 °C 45 s, 72 °C 90s). TP1 mRNA was amplified using primers TP1.1 and TP1.2 for 28 cycles (cycles the same as hTERT). Reaction products were resolved on an 8% polyacrylamide gel, stained with SYBR Green (Molecular Probes) and visualized by scanning on a Storm 860 (Molecular Dynamics). The results, shown in Figure 5, demonstrate that hTERT mRNA levels correlate directly with telomerase activity levels in the cells tested.

20

EXAMPLE 3

CHARACTERIZATION OF AN hTERT INTRONIC SEQUENCE

A putative intron was first identified by PCR amplification of human genomic DNA, as described in this example, and subsequently confirmed by sequencing the genomic clone λ G ϕ 5 (see Example 4). PCR amplification was carried out using the forward primer TCP1.57 paired individually with the reverse primers TCP1.46, TCP1.48, TCP1.50, TCP1.52, TCP1.54, TCP1.56, and TCP1.58 (see Table 2). The products from genomic DNA of the TCP1.57/TCP1.46, TCP1.48, TCP1.50, TCP1.52, TCP1.54, or TCP1.56 amplifications were approximately 100 basepairs larger than the products of the pGRN121 amplifications. The TCP1.57/TCP1.58 amplification was the same on either genomic or pGRN121 DNA. This indicated the genomic DNA contained an insertion between the sites for TCP1.58 and TCP1.50. The

30

PCR products of TCP1.57/TCP1.50 and TCP1.57/TCP1.52 were sequenced directly, without subcloning, using the primers TCP1.39, TCP1.57, and TCP1.49.

As shown below, the 104-base intronic sequence (SEQUENCE ID NO: 7) is inserted in the hTERT mRNA (shown in bold) at the junction corresponding to bases 274 and 275 of Figure 16:

```
CCCCCGCCGCCCCCTCCTTCCGCCAG/GTGGGCCTCCCCGGGGTCGGCG
TCCGGCTGGGGTTGAGGGCGGCCGGGGGAACCAGCGACATGCGGAGAGC
AGCGCAGGCGACTCAGGGCGCTTCCCCCGCAG/GTGTCTGCCTGAAGGA
GCTGGTGGCCCGAGTGCTGCAG
```

The "/" indicates the splice junctions; the sequence shows good matches to consensus 5' and 3' splice site sequences typical for human introns.

This intron contains motifs characteristic of a topoisomerase II cleavage site and a NF κ B binding site (see Figure 21). These motifs are of interest, in part, because expression of topoisomerase II is up regulated in most tumors. It functions to relax DNA by cutting and rewinding the DNA, thus increasing expression of particular genes. Inhibitors of topoisomerase II have been shown to work as anti-tumor agents. In the case of NF κ B, this transcription factor may play a role in regulation of telomerase during terminal differentiation, such as in early repression of telomerase during development and so is another target for therapeutic intervention to regulate telomerase activity in cells.

EXAMPLE 4

CLONING OF LAMBDA PHAGE G Φ 5 AND CHARACTERIZATION OF hTERT GENOMIC SEQUENCES

A. Lambda G Φ 5

A human genomic DNA library was screened by PCR and hybridization to identify a genomic clone containing hTERT RNA coding sequences. The library was a human fibroblast genomic library made using DNA from WI38 lung fibroblast cells (Stratagene, Cat # 946204). In this library, partial Sau3AI fragments are ligated into the XhoI site of Lambda FIX Φ II Vector (Stratagene), with an insert size of 9-22 kb.

The genomic library was divided into pools of 150,000 phage each, and each pool screened by nested PCR (outer primer pair TCP1.52 & TCP1.57; inner pair TCP1.49 & TCP1.50, see Table 1). These primer pairs span a putative intron (see Example 3, *supra*) in the genomic DNA of hTRT and ensured the PCR product was derived from a genomic source and not from contamination by the hTRT cDNA clone. Positive pools were further subdivided until a pool of 2000 phage was obtained. This pool was plated at low density and screened via hybridization with a DNA fragment encompassing basepairs 1552-2108 of Figure 16 (restriction sites SphI and EcoRV, respectively).

Two positive clones were isolated and rescreened via nested PCR as described above; both clones were positive by PCR. One of the clones (λ G Φ 5) was digested with NotI, revealing an insert size of approximately 20 kb. Subsequent mapping (see below) indicated the insert size was 15 kb and that phage G Φ 5 contains approximately 13 kb of DNA upstream from the start site of the cDNA sequence.

Phage G Φ 5 was mapped by restriction enzyme digestion and DNA sequencing. The resulting map is shown in Figure 7. The phage DNA was digested with NcoI and the fragments cloned into pBBS167. The resulting subclones were screened by PCR to identify those containing sequence corresponding to the 5' region of the hTRT cDNA. A subclone (pGRN140) containing a 9 kb NcoI fragment (with hTRT gene sequence and 4-5 kb of lambda vector sequence) was partially sequenced to determine the orientation of the insert. pGRN 140 was digested using SalI to remove lambda vector sequences, resulting in pGRN144. pGRN144 was then sequenced. The results of the sequencing are provided in Figure 21. The 5' end of the hTRT mRNA corresponds to base 2441 of Figure 21. As indicated in Figure 7, two Alu sequence elements are located 1700 base pairs upstream of the hTRT cDNA 5' end and provide a likely upstream limit to the promoter region of hTRT. The sequence also reveals an intron positioned at bases 4173 in Figure 21, 3' to the intron described in Example 3, *supra*.

B. Additional Genomic Clones

In addition to the genomic clone described above, two P1 bacteriophage

clones and one human BAC clone are provided as illustrative embodiments of the invention. P1 inserts are usually 75-100 kb, and BAC inserts are usually over 100 Kb.

The P1 clones (DMPC-HFF#1-477(F6) -GS #15371 and DMPC-HEF#1-1103(H6) -GS #15372) were obtained by PCR screening of a human P1 library derived from human foreskin fibroblast cells (Shepherd et al., 1994, *PNAS USA* 91:2629) using primers TCP1.12 and UTR2 which amplify the 3' end of hTRT. These clones were both negative (failed to amplify) with primers that amplify the 5' end of hTRT.

The human BAC clone (326 E 20) was obtained with a hybridization screen of a BAC human genomic library using an 1143 bp SphI/XmnI fragment of pGRN121 (Figure 16; bases 1552-2695) that encompasses the RT motif region. The clone is believed to include the 5' end of the gene. The hTRT genomic clones in this example are believed to encompass the entire hTRT gene.

EXAMPLE 5

CHROMOSOMAL LOCATION OF hTRT GENE

The hTRT gene was localized to chromosome 5p by radiation hybrid mapping (Boehnke et al., 1991, *Am J Hum Genet* 49:1174; Walter et al., 1994, *Nature Genet* 7:22) using the medium resolution Stanford G3 panel of 83 RH clones of the whole human genome (created at the Stanford Human Genome Center). A human lymphoblastoid cell line (donor; rM) was exposed to 10,000 rad of x-rays and was then fused with nonirradiated hamster recipient cells (A3). Eighty-three independent somatic cell hybrid clones were isolated, and each represents a fusion event between an irradiated donor cell and a recipient hamster cell. The panel of G3 DNA was used for ordering markers in the region of interest as well as establishing the distance between these markers.

The primers used for the RH mapping were TCP1.12 and UTR2 with amplification conditions of 94°C 45 sec, 55°C 45 sec, 72°C 45 sec, for 45 cycles using Boehringer Mannheim Taq buffer and Perkin-Elmer Taq. The 83 pools were amplified independently and 14 (17%) scored positive for hTRT (by appearance of a 346 bp band). The amplification results were submitted to Stanford RH server, which then

provided the map location, 5p, and the closest marker, STS D5S678.

By querying the Genethon genome mapping web site, the map location identified a YAC that contains the STS marker D5S678: CEPH YAC 780_C_3 Size: 390,660 kb. This YAC also contained chromosome 17 markers. This result indicated that the hTERT gene is on chromosome 5, near the telomeric end. There are increased copy numbers of 5p in a number of tumors. Cri-du-chat syndrome also has been mapped to deletions in this region.

EXAMPLE 6

10 DESIGN AND CONSTRUCTION OF VECTORS FOR EXPRESSION OF hTERT PROTEINS AND POLYNUCLEOTIDES

Expression of hTERT in Bacteria

The following portion of this example details the design of hTERT-expressing bacterial and eukaryotic cell expression vectors to produce large quantities of full-length, biologically active hTERT. Generation of biologically active hTERT protein in this manner is useful for telomerase reconstitution assays, assaying for telomerase activity modulators, analysis of the activity of newly isolated species of hTERT, identifying and isolating compounds which specifically associate with hTERT, analysis of the activity of an hTERT variant protein that has been site-specifically mutated, and as an immunogen, as a few examples.

pThioHis A/hTERT Bacterial Expression Vector

To produce large quantities of full-length hTERT, the bacterial expression vector pThioHis A (Invitrogen, San Diego, CA) was selected as an expression system. The hTERT-coding insert includes nucleotides 707 to 4776 of the hTERT insert in the plasmid pGRN121. This nucleotide sequence includes the complete coding sequence for the hTERT protein.

This expression vector of the invention is designed for inducible expression in bacteria. The vector can be induced to express, in *E. coli*, high levels of a fusion protein composed of a cleavable, HIS tagged thioredoxin moiety and the full length hTERT protein. The use of the expression system was in substantial accordance

with the manufacturer's instructions. The amino acid sequence of the fusion protein encoded by the resulting vector of the invention is shown below; (-*-) denotes an enterokinase cleavage site:

MSDKI IHLTDDSFDTDLKADGAILVDFWAHWCGPCKMIAPILDEIADEYQGKLTVAK
5 LRIDHNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSGSGDD
DDK - * - VPMHELEIFEFAAASTQRCVLLRTWEALAPATPAMPRAPRCRAVRSLRSHY
REVLPLATFVRRLGPQGWRLVQRGDPAAFRALVAQCLVCVPWDARPPPAAPSFRQVSC
LKELVARVLQRLCERGAKNVLAFGFALLDGARGGPPEAFTTSVRSYLPNTVTDALRGS
GAWGLLLRRVGDDVLVHLLARCALFVLVAPSCAYQVCGPPLYQLGAATQARPPPHASG
10 PRRRLGCERAWNHSVREAGVPLGLPAPGARRRGGSSASRLPLPKRPRRGAAPEPERTP
VGQGSWAHPGRTRGPSDRGFCVVSPARPAEEATSLEGALSGTRHSHPSVGRQHHAGPP
STSRPPRPWDTPCPPVYAETKHFYSSGDKEQLRPSFLLSSLRPSLTGARRLVETIFL
GSRPWMPGTPRRLPRLPQRYWQMRPLFLELLGNHAQCPYGVLLKTHCPLRAAVTPAAG
VCAREKPQGSVAAPEEEDTDPRRLVQLLRQHSSPWQVYGFVRACLRRLVPPGLWGSRH
15 NERRFLRNTKKFISLGKHAKLSLQELTWKMSVRDCAWLRRSPGVGCVPAAEHRLREEI
LAKFLHWLMSVFWVELLSFFYT.TETTFQKNRLFYRKSTWSKLSIGIRQHLKRVQL
RELSEAEVRQHREARPALTSRLRFIPKPDGLRPVWMDYVVGARTFRREKPAERLTS
RVKALFSVLNYERARRPGLLGASVLGLDDIHRWRTFVLRVRAQDPPPELYFVKVDVT
GAYDTIPQDRLTEVIASIIKPQNTYCVRRYAVVQKAAHGHVRKAFKSHVSTLTDLQPY
20 MRQFVAHLQETSPLRDAVVIEQSSSLNEASSGLFDVFLRFMCHHAVRIRGKSYVQCQG
IPQGSILSTLLCSLCYGD MENKLFAGIRRDGLLLRLVDDFLLVTPHLTHAKTFLRTL
RGVPEYGCVVNLRKTVVNFVVEDEALGGTAFVQMPAHGLFPWCGLLLDTRTLEVQSDY
SSYARTSIRASLTFNRGFKAGRNMRRKLFGLVRLKCHSLFLDLQVNSLQTVCTNIYKI
LLLQAYRFHACVLQLPFHQVWKNPTFFLRVISDTASLCYSILKAKNAGMSLGAKGAA
25 GPLPSEAVQWLCHQAFLKLTRHRVTYVPLLGSLRTAQTQLSRKLP GTTLTALEAAAN
PALPSDFKTILD

pGEX-2TK with hTRT Nucleotides 3272 to 4177 of pGRN121

This construct of the invention is used to produce fusion protein for,
30 e.g., the purpose of raising polyclonal and monoclonal antibodies to hTRT protein.
Fragments of hTRT can also be used for other purposes, such as to modulate

telomerase activity, for example, as a dominant-negative mutant or to prevent the association of a telomerase component with other proteins or nucleic acids.

To produce large quantities of an hTERT protein fragment, the *E. coli* expression vector pGEX-2TK (Pharmacia Biotech, Piscataway N.J) was selected, and used essentially according to manufacturer's instructions to make an expression vector of the invention. The resulting construct contains an insert derived from nucleotides 3272 to 4177 of the hTERT insert in the plasmid pGRN121. The vector directs expression in *E. coli* of high levels of a fusion protein composed of glutathione-S-transferase sequence (underlined below), thrombin cleavage sequence (double underlined), recognition sequence for heart muscle protein kinase (italicized), residues introduced by cloning in brackets ([GSVTK]) and hTERT protein fragment (in bold) as shown below:

MSPIIGYWKIKGLVOPTRLILEYLEEKYEELHYERDEGDKWRNKKFELGLEFPNLPYY
IDGDVKLTOSMAIIRYIADKHNMILGGCPKERAELSMLEGAVLDIRYGVSR IAYSKDFE
TLKVDFLSKLPMLKMEEDRLCHKTYLNGDHTVTHPDEMLYDALDVVLVMDPMCLDAFP
KLVCCKRIFAIPOIDKVLKSSKYIAWPLOGWOATEGGGDHPPKSDLVPRGSRRA SV
[GSVTK] IPQGSILSTLLCSLCYGD MENKLFAGIRRDGLLLRLVDDFLLVTPHLTHART
FLRTLVRGVPEYGCVVNLRKTVVNFVPEDEALGGTAFVQMPAHGLFPWCGLLLDTRTL
EVQSDYSSYARTSIRASVTFNRGFKAGRNMRRKLFGLRLKCHSLFLDLQVNSLQTVCL
TNIYKILLQAYRFHACVLQLPFFHQVWKNPTFFLRVISDTASLCYSILKAKNAGMSL
GARGAAGPLPSEAVQWLCHQAFLLKLTRHRVTYVPLLGLSLRTAQTL SRKLP GTTLTA
LEAAANPALPSDFKTILD

When this fusion protein was expressed, it formed insoluble aggregates. It was treated generally as described above, in the section entitled purification of proteins from inclusion bodies. Specifically, induced cells were suspended in PBS (20 mM sodium phosphate, pH 7.4, 150 mM NaCl) and disrupted by sonication. NP-40 was added to 0.1%, and the mixture was incubated for 30 minutes at 4°C with gentle mixing. The insoluble material was collected by centrifugation at 25,000g for 30 minutes at 4°C. The insoluble material was washed once in 4M urea in PBS, collected by centrifugation, then washed again in PBS. The collected pellet was estimated to contain greater than 75% fusion protein. This material was dried in a speed vacuum, then suspended in adjuvant for injection into mice and rabbits for the generation of antibodies. Separation of the recombinant protein from the glutathione S-transferase moiety is accomplished by site-specific

proteolysis using thrombin according to manufacturer's instructions.

pGEX-2TK with hTRT Nucleotides 2426 to 3274 of pGRN121 with HIS-8 Tag

To produce large quantities of a fragment of hTRT, another *E. coli* expression vector pGEX-2TK construct was prepared. This construct contains an insert derived from nucleotides 2426 to 3274 of the hTRT insert in the plasmid pGRN121 and a sequence encoding eight consecutive histidine residues (HIS-8 Tag). To insert the HIS-8 TAG, the pGEX-2TK vector with hTRT nucleotides 2426 to 3274 of pGRN121 was linearized with BamH1. This opened the plasmid at the junction between the GST-thrombin-heart muscle protein kinase and the hTRT coding sequence. A double stranded oligonucleotide with BamH1 compatible ends was ligated to the linearized plasmid resulting in the in-frame introduction of eight histidine residues upstream of the hTRT sequence.

The vector directs expression in *E. coli* of high levels of a fusion protein composed of glutathione-S-transferase sequence (underlined); thrombin cleavage sequence (double underlined); recognition sequence for heart muscle protein kinase (italicized); a set of three and a set of five residues introduced by cloning are in brackets ([GSV] and [GSVTK]); eight consecutive histidines (also double underlined); and hTRT protein fragment (in bold):

MSPILGYWKIKGLVOPTRLILLEYLEEKYEHLYERDEGDKWRNKKFELGLEFP
NLPYYIDGDVKLTOSMAIIRYIADKHNMLGCGPKERAISMLEGAVLDIRYGVS
RIAYSKDFETLKVDLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDEFMLYDAL
DVVLVMDPMCLDAFPKLVCFKKRIEAIPOIDKYLKSSKYIAWPLOGWOATEGG
GDHPPKSDLVPRGSRRASV[GSV]HHHHHHHH[GSVTK]MSVYVVELLSFFYV
TETTFQKNRLFFYRPSVWSKLQSIGIRQHLKRVQLRELSEAEVRQHREARP
ALLTSRLRFIPKPDGLRPIVNMDYVVGARTFRREKRAERLTSRVKALFSVL
NYERARRPGLLGASVLGLDDIHRWRTFVLRVRAQDPPELYFVKVDVTG
AYDTIPQDRLTEVIASIIKPQNTYCVRRYAVVQKAAHGHVRKAFKSHVSTL
TDLOPYMROQFVAHLQETSPLRDAVVIEQSSSLNEASSGLFDVFLRFMCHHA
VRIRGKSYVQCQGI

Each of the pGEX-2TK vectors of the invention can be used to produce fusion protein for the purpose of raising polyclonal and monoclonal antibodies to hTRT protein. Additionally, this fusion protein can be used to affinity purify antibodies raised to hTRT peptides that are encompassed within the fusion protein. Separation of

the recombinant protein from the glutathione S-transferase moiety can be accomplished by site-specific proteolysis using thrombin according to manufacturer's instructions.

pGEX-2TK with hTRT Nucleotides 2426 to 3274 of pGRN121, no HIS-8 Tag

5 To produce large quantities of a fragment of hTRT, another *E. coli* expression vector pGEX-2TK construct was prepared.

This construct contains an insert derived from nucleotides 2426 to 3274 of the hTRT insert in the plasmid pGRN121, but without the HIS-8 tag of the construct described above. The vector directs expression in *E. coli* of high levels of a fusion protein composed of glutathione-S-transferase (underlined), thrombin cleavage sequence (double underlined), recognition sequence for heart muscle protein kinase (italicized), residues introduced by cloning in brackets ([GSVTK]) and hTRT protein fragment (in bold):

15 MSPILGYWKIKGLVOPTRLLLEYLEEKYEHLYERDEGDKWRNKKFELGLEFPNLPYY
IDGDVKLTOSMAIIRYIADKHNMLGGCPKERAEISMLEGAVLDIRYGVSRIAYSKDFE
TLKVDFELSKLPEMLKMFEDRLCHKTYLNGDHVTHPDEMLYDALDWVLVCDPMCLDAFP
KLWCEKRIEALPQIDKYLKSSKVIAWPLOGWOSTEGGGDEHPKSDWERGSRRASV
[GSVTK] MSVYVVELLRSFFYVTETTPQKNRLFFYRPSVWSKLQSIGIRQHLKRVQLRE
LSEAEVRQHREARPALLTSRLRFIPKPDGLRPVNM DYVVGARTFRREKRAERLTSRK
20 ALFSVLNYERARRPGLLGASVLGLDDIHRWRTFVLRVRAQDPPPEYFVKVDVTGAYD
TIPQDRLTEVIASIIKPQNTYCVRRYAVVQKAAHGVRKAFKSHVSTLTDLQPYMRQFV
AHLQETSPLRDAVVIEQSSSLNEASGLFDVFLRFMCHHAVRIRGKSYVQCQGI

pGEX-2TK with hTRT Nucleotides 1625 to 2458 of pGRN121

25 To produce large quantities of a fragment of hTRT protein, another *E. coli* expression vector pGEX-2TK construct was prepared.

This construct contains an insert derived from nucleotides 1625 to 2458 of the hTRT insert in the plasmid pGRN121. The vector directs expression in *E. coli* of high levels of a fusion protein composed of glutathione-S-transferase, (underlined), thrombin cleavage sequence (double underlined), recognition sequence for heart muscle protein kinase (italicized) residues introduced by cloning in brackets ([GSVTK]) and hTRT protein fragment (in bold):

30 MSPILGYWKIKGLVOPTRLLLEYLEEKYEHLYERDEGDKWRNKKFELGLEFP
NLPYYIDGDVKLTOSMAIIRYIADKHNMLGGCPKERAEISMLEGAVLDIRYGV
35 SRIAYSKDFETLKVDFELSKLPEMLKMFEDRLCHKTYLNGDHVTHPDEMLYDA

LDVVLYMDPMCLDAFPKLYCFKKRIEAIPOIDKYLKSSKYIAWPLOGWOATEFG
GGDHPPKSDLVPRGSRRASV[GSVTK]ATSLEGALSGTRHSHPSVGRQHHAGP
PSTSRPPRPWDTPCPPVYAETKHFYSSGDKEQLRPSFLLSSLRPSLTGARR
LVETIFLGSRPWMPGTPRRLPRLPQRYWQMRPLFLELLGNHAQCPYGVLL
5 KTHCPLRAAVTPAAGVCAREKPQGSVAAPEEEDTDPRLVQLLRQHSSPW
QVYGFVRACLRRLLVPPGLWGSRHNERFLRNTKKFISLGKHAKLSLQELT
WKMSVRDCAWLRSPGVGCVPAAEHRLREEILAKFLHWLMSVYVVELLR
S

10 *pGEX-2TK with hTRT Nucleotides 782 to 1636 of pGRN121*

To produce large quantities of a fragment of hTRT protein, another *E. coli* expression vector pGEX-2TK construct was prepared.

This construct contains an insert derived from nucleotides 782 to 1636 of the hTRT insert in the plasmid pGRN121. The vector directs expression in *E. coli* of high levels of a fusion protein composed of glutathione-S-transferase, (underlined), thrombin cleavage sequence (double underlined), recognition sequence for heart muscle protein kinase (italicized) residues introduced by cloning in brackets ([GSVTK]) and hTRT protein fragment (in bold):

20 MSPLGVWKAHGLVQPTRLLLEYLSPKVEEHLVERDEGDKWRNKKFELGLEFPNLPVY
LDSDHHLTOSMAITSPYADKHNLGGCPKEPAEISMLEGAVLDIRYGVSRJAYSKDDE
TLKVDLISKLPDEMLKVFEDRLCHKTYLNGDHVTHPDDEMLYDALDVVLYMDPMCLDAEP
KLVCFKKRIEAIPQIDKYLKSSKYIAWPLOGWOATEFGGDHPPKSDLVPRGSRRASV[
GSVTK]MPRAPRCRAVRSLLSHYREVLPATFVRRLGPGQWRLVQRGDPAAFRALVAQ
CLVCVPWDARPPAAPSFQVSCLELVARVLQRLCERGAKNVLAFGFALLDGARGGPP
25 EATTSVRSYLPNTVTDALRGSGAWGLLLRRVGDDVLVHLLARCALFVLVAPCAYQVCG
PPLYQLGAATQARPPPHASGPRRLGCEAWNHSVREAGVPLGLPAPGARRRGGSASR
SLPLPKRPRRGAAPEPERTPVGQGSWAHPGRTRGPSDRGFCVVSPARPAEEATSL

pT7FLhTRT with hTRT cDNA Lacking 5'-Non-Coding Sequence

30 As described above, in one embodiment, the invention provides for an hTRT that is modified in a site-specific manner to facilitate cloning into bacterial, mammalian, yeast and insect expression vectors without any 5' untranslated hTRT sequence. In some circumstances, minimizing the amount of non-protein encoding sequence allows for improved protein production (yield) and increased mRNA
35 stability. In this embodiment of the invention, the hTRT gene's 5' non-coding region was removed before cloning into a bacterial expression vector.

This was effected by engineering an additional restriction endonuclease

site just upstream (5') to the start (ATG) codon of the hTRT coding sequence (Figure 16). The creation of a restriction site just 5' to the coding region of the protein allows for efficient production of a wide variety of vectors that encode fusion proteins, such as fusion proteins comprising labels and peptide TAGs, for immunodetection and purification.

Specifically, the oligonucleotide

5'- CCGGCCACCCCCCATATGCCGCGCGCTCCC-3' was used as described above to modify hTRT cDNA nucleotides 779 to 781 of the hTRT cDNA (Figure 16) from GCG to CAT. These 3 nucleotides are the last nucleotides before the ATG start codon so they do not modify the protein sequence. The change in sequence results in the creation of a unique NdeI restriction site in the hTRT cDNA. Single-stranded hTRT DNA was used as a DNA source for the site directed mutagenesis. The resulting plasmid was sequenced to confirm the success of the mutagenesis.

This modification allowed the construction of the following plasmid of the invention, designated pT7FLhTRT. The site-specifically modified hTRT sequence (addition of the NdeI restriction site) was digested with NdeI and NotI

to generate an hTRT encoding

nucleic acid fragment. The fragment was then cloned into a pSL3418 plasmid previously restriction digested with NdeI and SmaI (also a blunt ended cutter). pSL 3418 is a modified pAED4 plasmid into which a FLAG sequence (Immunex Corp, Seattle WA) and an enterokinase sequence are inserted just upstream from the above-referenced NdeI site. This plasmid, designated pT7FLhTR, allows the expression of full length hTRT (with a Flag-Tag at its 5' end) in an E.coli strain expressing the T7 RNA polymerase.

Plasmids with hTRT cDNA Lacking 3'-Non-Coding Sequence

As discussed above, the invention provides for expression vectors containing TRT-encoding nucleic acids in which some or all non-coding sequences have been deleted. In some circumstances, minimizing the amount of non-protein encoding sequence allows for improved protein production (yield) and increases mRNA stability. In this embodiment of the invention, the 3' untranslated region of hTRT is deleted before cloning into a bacterial expression plasmid.

The plasmid pGRN121, containing the full length hTRT cDNA, as discussed above, was first deleted of all *Apal* sites. This was followed by deletion of the *MscI*-*HincII* hTRT restriction digest enzyme fragment containing the 3'UTR. The *NcoI*-*XbaI* restriction digest fragment containing the stop codon of hTRT was then
5 inserted into the *NcoI*-*XbaI* site of pGRN121 to make a plasmid equivalent to pGRN121, designated pGRN124, except lacking the 3'UTR.

Bacterial Expression Vectors Using Antibiotic Selection Markers

The invention also provides for bacterial expression vectors that can
10 contain selection markers to confer a selectable phenotype on transformed cells and sequences coding for episomal maintenance and replication such that integration into the host genome is not required. For example, the marker may encode antibiotic resistance, particularly resistance to chloramphenicol (see Harrod (1997) *Nucleic Acids Res.* 25: 1720-1726), kanamycin, G418, bleomycin and hygromycin, to permit selection
15 of those cells transformed with the desired DNA sequences, see for example, Blondelet-Rouault (1997) *Gene* 190:315-317; and Mahan (1995) *Proc Natl Acad Sci U S A* 92:669-673.

In one embodiment of the invention, the full length hTRT was cloned into a modified BlueScript plasmid vector (Stratagene, San Diego, CA), designated
20 pBBS235, into which a chloramphenicol antibiotic resistance gene had been inserted. The *NotI* fragment from pGRN124 (discussed above) containing the hTRT ORF into the *NotI* site of pBBS235 so that the TRT ORF is in the opposite orientation of the vector's Lac promoter. This makes a plasmid that is suitable for mutagenesis of plasmid inserts, such as TRT nucleic acids of the invention. This plasmid construct, designated
25 pGRN125, can be used in the methods of the invention involving mutagenesis of telomerase enzyme and TRT protein coding sequences and for *in vitro* transcription of hTRT using the T7 promoter (and *in vitro* transcription of antisense hTRT using the T3 promoter).

In another embodiment of the invention, *NotI* restriction digest
30 fragments from pGRN124 containing the hTRT ORF were subcloned into the *NotI* site of pBBS235 (described above) so the TRT ORF is in the same orientation as the

vector's Lac promoter. This makes a plasmid, designated pGRN126, that can be used for expression of full length hTERT in *E. coli*. The expressed product will contain 29 amino acids encoded by the vector pBBS235, followed by 18 amino acids encoded by the 5'UTR of hTERT, followed by the full length hTERT protein.

5 In a further embodiment of the invention, *in vitro* mutagenesis of pGRN125 was done to convert the hTERT initiating ATG codon into a Kozak consensus and create EcoRI and BglII restriction digest sites to facilitate cloning into expression vectors. The oligonucleotide

5'-

10 TGCGCACGTGGGAAGCCCTGGCagatctgAattCcaCcaTGCCGCGCGCTCCCCGC
TG-3' (altered nucleotides in lower case) was used in the mutagenesis procedure. The resulting expression vector was designated pGRN127.

In another embodiment of the invention, the second Asp of the TRT "DD motif" was converted to an alanine to create a non-functional telomerase enzyme, thus creating a mutant TRT protein for use as a dominant/negative mutant. The hTERT coding sequence was mutagenized *in vitro* using the oligonucleotide 5'-
15 CGGGACGGGCTGCTCCTGCGTTTGGTGGAcGcgTTCTTGTTGGTGACACCTCA
CCTCACC-3' to convert the asparagine codon for residue 869 (Asp869) to an alanine (Ala) codon. This also created an MluI restriction enzyme site. The resulting
20 expression plasmid was designated pGRN130, which also contains the Kozak consensus sequence as described for pGRN127.

25

The invention also provides a vector designed to express an antisense sequence fragment of hTERT. The pGRN126 plasmid was cut to completion with MscI and SmaI restriction enzymes and religated to delete over 95% of the hTERT ORF. One SmaI-MscI fragment was re-inserted during the process to recreate CAT activity. This
30 unpurified plasmid was then redigested with Sall and EcoRI and the fragment containing the initiating codon of the hTERT ORF was inserted into the Sall-EcoRI sites

of pBBS212 to make an antisense expression plasmid expressing the antisense sequence spanning the 5'UTR and 73 bases pair residues of the hTERT ORF (in mammalian cells). This plasmid was designated pGRN135.

5 Expression of hTERT Telomerase in Yeast

The present invention also provides hTERT-expressing yeast expression vectors to produce large quantities of full-length, biologically active hTERT.

Pichia pastoris Expression Vector pPICZ B and Full Length hTERT

10 To produce large quantities of full-length, biologically active hTERT, the *Pichia pastoris* expression vector pPICZ B (Invitrogen, San Diego, CA) was selected. The hTERT-coding sequence insert was derived from nucleotides 659 to 4801 of the hTERT insert in plasmid pGRN121. This nucleotide sequence includes the full-length sequence encoding hTERT. This expression vector is designed for inducible expression
15 in *P. pastoris* of high levels of full-length, unmodified hTERT protein. Expression is driven by a yeast promoter, but the expressed sequence utilizes the hTERT initiation and termination codons. No exogenous codons were introduced by the cloning. The resulting pPICZ B/hTERT vector was used to transform the yeast.

20 Pichia pastoris Expression Vector hTERT-His6/pPICZ B

A second *Pichia pastoris* expression vector of the invention derived from pPICZ B, also contains the full-length sequence encoding hTERT derived from nucleotides 659 to 4801 of the hTERT insert in the plasmid pGRN121. This hTERT-His6/pPICZ B expression vector encodes full length hTERT protein fused at its
25 C-terminus to the Myc epitope and His6 reporter tag sequences. The hTERT stop codon has been removed and replaced by vector sequences encoding the Myc epitope and the His6 reporter tag as well as a stop codon. This vector is designed to direct high-level inducible expression in yeast of the following fusion protein, which consists of hTERT sequence (underlined), vector sequences in brackets ([L] and [NSAVD]) the Myc
30 epitope (double underlined), and the His6 tag (italicized):

MPRAPRCRAVRSLLRSHYREVLPLATEFVRRLLGPOGWRLVORGDPAAFRALVA

OCLVCVPWDARPPPAAPSFROVSCLKELVARVLORLCERGAKNVLAFGFALL
 DGARGGPPEAFTTSVRSYLPNTVTDALRGSGAWGLLLRRVGDDVLVHLLARC
 ALFVLVAPSCAYOVCGPPLYOLGAATOARPPPHASGPRRRLGCERAWNHSVR
 EAGVPLGLPAPGARRRGGSASRSLPLPKRPRRGAAPEPERTPVGOGSWAHPGR
 5 TRGPSDRGFCVVSPARPAEEATSLEGALSGTRHSHPSVGROHHAGPPSTSRPPR
 PWDTPCPPVYAETKHFLYSSGDKEOLRPSFLLSSLRPSLTGARRLVETIFLGSRP
 WMPGTPRRLPRLPORYWOMRPLFLELLGNHAOCYPYGVLLKTHCPLRAAVTPA
 AGVCAREKPOGSVAAPEEEDTDPRLVOLLROHSSPWOVYGEVRACLRRLVP
 PGLWGSRHNERFLRNTKKFISLGKHAKLSLOELTWKMSVRDCAWLRRSPGV
 10 GCVPAAEHRLREEILAKFLHWLMSVYVVELLRSEFFYVTETTFOKNRLEFFYRKS
 VWSKLOSIGIROHLKRVOLRELSEAEVROHREARPALLTSRLRFIPKPDGLRPIV
 NMDYVVGARTERREKRAERLTSRVKALFSVLNYERARRPGLLGASVLGLDDIH
 RAWRTFVLRVRAODPPPELYFVKVDVTGAYDTIPODRLTEVIASIIKPONTYCV
 RRYAVVOKAAHGHVRKAFAKSHVSTLTDLOPYMROFVAHLOETSPLRDVAVIE
 15 QSSSLNEASSGLEDFVFLRFMCHHAVRIRGKSYVOCOIGIPOGSILSTLLCSLCYGD
 MENKLEAGIRRDGLLLRLVDDFLVTPHLTHAKTFLRTLVRGVPEYGCVVNLR
 KTVVNFPEDEALGGTAFVOMPAHGLFPWCGLLLDTRTLEVQSDYSSYARTSI
 RASLTFNRGEKAGRNMRRKLEFVIRLKCHSLFLDIQVNSLOTVCTNIYKILLLO
 AYRFHACVLQLPFHQOVWKNPTFFLRVISDTASLCYSILKAKNAGMSLGAKGA
 20 AGPLPSEAVOWLCHOAFLKLTRHRVTYVPLLGSRLTAOTQLSRKLPQTTLTA
 LEAAANPALPSDFKTILD[L]EOKLISEEDL[NSAVD]HHHHHH

25

Expression of hTERT in Insect Cells

The present invention also provides hTERT telomerase-expressing insect
 cell expression vectors that produce large quantities of full-length, biologically active
 30 hTERT.

Baculovirus Expression Vector pVL1393 and Full Length hTERT

The telomerase coding sequence of interest was cloned into the
 baculovirus expression vector pVL1393 (Invitrogen, San Diego, CA). This construct
 35 was subsequently cotransfected into *Spodoptera frugiperda* (sf-9) cells with linearized
 DNA from *Autographa californica* nuclear polyhedrosis virus (Baculogold-AcMNPV).
 The recombinant baculoviruses obtained were subsequently plaque purified and
 expanded following standard protocols.

This expression vector provides for expression in insect cells of high levels of full-length hTRT protein. Expression is driven by a baculoviral polyhedrin gene promoter. No exogenous codons were introduced by the cloning.

Baculovirus Expression Vector pBlueBacHis2 B and Full Length hTRT

5 To produce large quantities of full-length, biologically active hTRT, the baculovirus expression vector pBlueBacHis2 B (Invitrogen, San Diego, CA) was selected as a source of control elements. The hTRT-coding insert consisted of nucleotides 707 to 4776 of the hTRT insert in plasmid pGRN121.

A full length hTRT with a His6 and Anti-Xpress tags (Invitrogen) was also constructed. This vector also contains an insert consisting of nucleotides 707 to 4776 of the hTRT insert from the plasmid pGRN121. The vector directs expression in insect cells of high levels of full length hTRT protein fused to a cleavable 6-histidine and Anti-Xpress tags, and the amino acid sequence of the fusion protein is shown below; (-*-) denotes enterokinase cleavage site:

15 MPRGSHHHHHHGMASMTGGQQMGRDLYDDDDL*-DPSSRSAAGTME
FAAASTQRCVLLRTWEALAPATPAMPRAAPRCRAVRSLLRSHYREVLPLATFVR
RLGPQGWRLVQRGDPAAFRALVAQCLVCVPWDARPPPAAPSFRQVSCLKELV
ARVLQRLCERGAKNVLAFGFALLDGARGGPPEAFTTSVRSYLPNTVTDALRGS
GAWGLLLRRVGDDVLVHLLARCALFVLVAPSCAYQVCGPPLYQLGAATQARP
20 PPHASGPRRLGCERAWNHSVREAGVPLGLPAPGARRRGGSASRSLPLPKRPR
RGAAPERTPVGQGSWAHPGRTRGPSDRGFCVVSAPARPAEEATSLEGALSGT
RHSHPVGRQHHAGPPSTSRPPRPWDTPCPPVYAETKHFLYSSGDKEQLRPSFL
LSSLRPSLTGARRLVETIFLGSRPWPMPGTTPRRLPRLPQRYWQMRPLFLELLGNH
AQCPYGVLLKTHCPLRAAVTPAAGVCAREKPQGSVAAPEEEDTDPRLVQLLR
25 QHSSPWQVYGFVRACLRRLVPPGLWGSRHNERFLRNTKKFISLGKHAKLSLQ
ELTWKMSVRDCAWLRRSPGVGCVPAAEHRLREEILAKFLHWLMSVYVVELLR
SFFYVTETTFQKNRLFFYRKSVWSKLQSIGIRQHLKRVQLRELSEAEVRQHREA
RPALLTSRLRFIPKPDGLRPIVNM DYVVGARTFRREKRAERLTSRVKALFSVLN
YERARRPGLLGASVLGLDDIHRAWRTFVLRVRAQDPPPELYFVKVDVTGAYD
30 TIPQDRLTEVIASIIKPQNTYCVRRYAVVQKAAHGHVRKAFKSHVSTLTDLQPY
MRQFVAHLQETSPLRDAVVIEQSSSLNEASSGLFDVFLRFMCHHAVRIRGKSY
VQCQGIQGSILSTLLCSLCYGD MENKLFAGIRRDGLLLRLVDDFLLVTPHLTH
AKTFLRTLVRGVPEYGCVVNLKRTVVNFPVEDEALGGTAFVQMPAHGLFPWC
GLLLDTRTLEVQSDYSSYARTSIRASLTFNRGFKAGRNMRRKLFGLVRLKCHS
35 LFLDLQVNSLQTVCTNIYKILLLQAYRFHACVLQLPFHQVWKNPTFFLRVISD
TASLCYSILKAKNAGMSLGAKGAAGPLPSEAVQWLCHQAFLKLTRHRVTYV
PLLGSLRTAQTL SRKLP GTTLTALEAAANPALPSDFKTILD

Baculovirus Expression Vector pBlueBac4.5 and Full Length hTRT Protein

To produce large quantities of full-length, biologically active hTRT, a second baculovirus expression vector, pBlueBac4.5 (Invitrogen, San Diego, CA) was constructed. The hTRT-coding insert also consisted of nucleotides 707 to 4776 of the hTRT from the plasmid pGRN121.

5

Baculovirus Expression Vector pMelBacB and Full Length hTRT Protein

To produce large quantities of full-length, biologically active hTRT, a third baculovirus expression vector, pMelBacB (Invitrogen, San Diego, CA) was constructed. The hTRT-coding insert also consists of nucleotides 707 to 4776 of the
10 hTRT insert from the plasmid pGRN121.

pMelBacB directs expression of full length hTRT in insect cells to the extracellular medium through the secretory pathway using the melittin signal sequence. High levels full length hTRT are thus secreted. The melittin signal sequence is cleaved upon excretion, but is part of the protein pool that remains intracellularly. For that
15 reason, it is indicated in parentheses in the following sequence. The sequence of the fusion protein encoded by the vector is shown below:

(MKFLVNVALVFMVVYISYIYA)-DPSSRSAAGTMEFAAASTQRCVLL
RTWEALAPATPAMPAPRCRAVRSLRSHYREVLPLATFVRRLGPQGWRLVQ
RGDPAAFRALVAQCLVCVPWDARPPPAAPSFQVSCLELVARVLQRLCERG
20 AKNVLAFGFALLDGARGGPPEAFTTSVRSYLPNTVTDALRGSGAWGLLLRRV
GDDVLVHLLARCALFVLVAPSCAYQVCGPPLYQLGAATQARPPPHASGPRRRL
GCERAWNHSVREAGVPLGLPAPGARRRGGSASRSLPLPKRPRRGAAPERTP
VGQGSWAHPGRTRGPSDRGFCVVSAPPAEEATSLEGALSGTRHSHPSVGRQH
HAGPPSTSRRPPRWDTPCPPVYAETKHFYSSGDKEQLRPSFLLSSLRPSLTGAR
25 RLVTIFLGSRPWMPGTPRRLPRLPQRYWQMRPLFLELLGNHAQCPYGVLLKT
HCPLRAAVTPAAGVCAREKPQGSVAAPPEEDTDPRRLVQLLRQHSSPWQVYG
FVRACLRLVPPGLWGSRHNERFLRNTKKFISLGKHAKLSLQELTWKMSVRD
CAWLRSPGVGCVPAAEHRLREEILAKFLHWLMSVYVVELLSFFYVTETTFQ
KNRLFFYRKSVWSKLQSIGIRQHLKRVQLRELSEAEVRQHREARPALLTSRLRF
30 IPKPDGLRPIVNM DYVVGARTFRREKRAERLTSRVKALF SVLN YERARRPGLL
GASVLGLDDIHRAWRTFVLVR AQDPPPELYFVKVDVTGAYDTIPQDRLTEVI
ASIIKPQNTYCVRRYAVVQKAAHGHVRKAFKSHVSTLTDLPYMRQFVAHLQ
ETSPLRD AVVIEQSSSLNEASSGLFDVFLRFMCHHAVRIRGKSYVQCQGI PQGSI
LSTLLCSLCYGD MENKLFAGIRRDG LLLRLVDDFLLVTPHLTHAKTFLRTLVRG
35 VPEYGC VVNLRKTVVNFPVEDEALGGTAFVQMPAHGLFPWCGLLLDTRTLEV
QSDYSSYARTSIRASLT FNRGFKAGRNMRKLFGLVRLKCHSLFDLQVNSLQ
TVCTNIYKILLQAYRFHACVLQLPFHQVWKNPTFFLRVISDTASLCYSILKA
KNAGMSLGAKGAAGPLPSEAVQWLCHQAFLLKLTRHRVTYVPLLGSLRTAQT

QLSRKLP GTTLTALEAAANPALPSDFKTILD

Expression of hTERT in Mammalian Cells

5 The present invention also provides vectors to produce hTERT in large quantities as full-length, biologically active protein in a variety of mammalian cell lines, which is useful in many embodiments of the invention, as discussed above.

MPSV-hTERT Expression Plasmids

10 The invention also provides for an expression system for use in mammalian cells that give the highest possible expression of recombinant protein, such as telomerase, without actually modifying the coding sequence (e.g. optimizing codon usage). In one embodiment, the invention provides MPSV mammalian expression plasmids (from plasmid pBBS212, described as pMPSV-TM in Lin J-H (1994) Gene
15 47:287-292) capable of expressing the TRTs of the invention. The MPSV plasmids can be expressed either as stable or transient clones.

 In this expression system, while the hTERT coding sequence itself is unchanged, exogenous transcriptional control elements are incorporated into the vector. The myeloproliferative sarcoma virus (MPSV) LTR (MPSV-LTR) promoter, enhanced
20 by the cytomegalovirus (CMV) enhancer, is incorporated for transcriptional initiation. This promoter consistently shows higher expression levels in cell lines (see Lin J-H (1994) *supra*). A Kozak consensus sequence can be incorporated for translation initiation (see Kozak (1996) *Mamm. Genome* 7:563-574). All extraneous 5' and 3' untranslated hTERT sequences can be removed to insure that these sequences do not
25 interfere with expression, as discussed above. The MPSV plasmid containing the complete hTERT coding sequence, with all extraneous sequences included, is designated pGRN133. A control, hTERT "antisense" plasmid was also constructed. This vector is identical to pGRN133 except that the TRT insert is the antisense sequence of hTERT (the antisense, which control can be used as a vector is designated
30 pGRN134). The MPSV plasmid containing the complete hTERT coding sequence with all other extraneous sequences removed and containing the Kozak consensus sequence is designated pGRN145.

 Two selection markers, PAC (Puromycin-N-acetyl-transferase = Puromycin resistance) and HygB (Hygromycin B = Hygromycin resistance) are present

for selection of the plasmids after transfection (see discussion referring to selectable markers, above). Double selection using markers on both sides of the vector polylinker should increase the stability of the hTERT coding sequence. A DHFR (dihydrofolate reductase) encoding sequence is included to allow amplification of the expression cassette after stable clones are made. Other means of gene amplification can also be used to increase recombinant protein yields.

The invention also provides for MPSV mammalian expression plasmids containing hTERT fusion proteins. In one embodiment, the hTERT sequence, while retaining its 5' untranslated region, is linked to an epitope flag, such as the IBI FLAG (International Biotechnologies Inc. (IBI), Kodak, New Haven, CT) and inserted into the MPSV expression plasmid (designated pGRN147). This particular construct contains a Kozak translation initiation site. The expressed fusion protein can be purified using the M-1 anti-FLAG octapeptide monoclonal antibody (IBI, Kodak, supra).

In another embodiment, hTERT is site-specifically altered. One amino acid residue codon is mutagenized, changing the aspartic acid at position 869 to an alanine. This Asp869->Ala hTERT mutant, retaining its 5' untranslated region and incorporating a Kozak sequence, was inserted into an MPSV expression plasmid, and designated pGRN146. The Asp869->Ala hTERT mutant was further engineered to contain the FLAG sequence, as described above, and the insert cloned into an MPSV expression plasmid. This expression plasmid is designated pGRN154. Specifically, for pGRN154, an Eam1105I restriction digest fragment from pGRN146 containing the Kozak sequence-containing "front end" (5' segment) of hTERT is cloned into the Eam1105I sites of pGRN147 (see above) to make an MPSV expression plasmid capable of expressing hTERT with a Kozak sequence, the above-described D869->A mutation, and the IBI flag.

Another embodiment of the invention is an expression plasmid derived from pGRN146. The mammalian expression plasmid, designated pGRN152, was generated by excising the EcoRI fragment from plasmid pGRN146 (containing the hTERT ORF) and cloned into the EcoRI site of pBBS212 to remove the 5'UTR of hTERT. The hTERT is oriented so that its expression is controlled by the MPSV promoter. This makes a mammalian expression plasmid that expresses hTERT with a Kozak consensus

sequence and the D869->A mutation, and uses the MPSV promoter.

The invention provides for a mammalian expression vector in which hTERT is oriented so that the hTERT coding sequence is driven by the MPSV promoter. For example, an EcoR1 restriction digest fragment from pGRN137 containing the hTERT open reading frame (ORF) was cloned into the EcoR1 site of pBBS212 (see below), thus removing the 5' untranslated region (5'-UTR) of hTERT. pGRN137 was constructed by excising a Sall-Sse8387I fragment from pGRN130, described below, containing the Kozak mutation of hTERT into the Sal 1-SSE 8387I sites of pGRN136, making a mammalian expression plasmid expressing hTERT containing a Kozak consensus sequence off the MPSV promoter. Plasmid pGRN136 was constructed by excising a HindIII Sall fragment from pGRN126 containing the hTERT ORF and cloning it into the HindIII Sall sites of plasmid, pBBS242, making a mammalian expression plasmid expressing hTERT off the MPSV promoter). This makes a mammalian expression plasmid, designated pGRN145, that expresses hTERT with a Kozak consensus sequence using the MPSV promoter. See also the pGRN152 MPSV promoter-driven mammalian expression vector described below.

hTERT Expressed in 293 Cells using Episomal Vector pEBVHis

An episomal vector, pEBVHis (Invitrogen, San Diego, CA) was engineered to express an hTERT fusion protein comprising hTERT fused to an N-terminal extension epitope tag, the Xpress epitope (Invitrogen, San Diego, CA) (designated pGRN122). The NotI hTERT fragment from pGRN121 containing the hTERT ORF was cloned into the NotI site of pEBVHisA so that the hTERT ORF is in the same orientation as the vector's Rous Sarcoma Virus (RSV) promoter. In this orientation the His6 flag was relatively closer to the N-terminus of hTERT.

A vector was also constructed containing as an insert the antisense sequence of hTERT and the epitope tag (the plasmid designated pGRN123, which can be used as a control). The vector was transfected into 293 cells and translated hTERT identified and isolated using an antibody specific for the Xpress epitope. pEBVHis is a hygromycin resistant EBV episomal vector that expresses the protein of interest fused to a N-terminal peptide. Cells carrying the vector are selected and expanded, then

nuclear and cytoplasmic extracts prepared. These and control extracts are immunoprecipitated with anti-Xpress antibody, and the immunoprecipitated beads are tested for telomerase activity by conventional assay.

5 **Expression Recombinant hTERT in Mortal, Normal Diploid Human Cells**

In one embodiment of the invention, recombinant hTERT and necessary telomerase enzyme complex components can be expressed in normal, diploid mortal cells to increase their proliferative capacity or to immortalize them, or to facilitate immortalizing them. This allows one to obtain diploid immortal cells with an otherwise normal phenotype and karyotype. As discussed above, this use of telomerase
10 has enormous commercial utility.

Sense hTERT (Figure 16) and antisense hTERT) were cloned into a CMV vector. These vectors were purified and transiently transfected into two normal, mortal, diploid human cell clones. The human clones were young passage diploid
15 human BJ and IMR90 cell strains.

Analysis of telomerase activity using a TRAP assay (utilizing the TRAPeze™ Kit (Oncor, Inc., Gaithersburg, MD) showed that transfection of sense hTERT - but not antisense hTERT - generated telomerase activity in both the BJ and IMR90 cell strains.

20

Expression of Recombinant hTERT in Immortalized IMR90 Human Cells

Using the same hTERT sense construct cloned into CMV vectors used in the above described diploid human BJ and IMR90 cell strains studies, immortalized SW13 ALT pathway cell line (an IMR90 cell immortalized with SV40 antigen) was transiently transfected. A TRAP assay (TRAPeze, Oncor, Inc, Gaithersburg, MD)
25 demonstrated that telomerase activity was generated in the sense construct transfected cells.

30 **Vectors for Regulated Expression of hTERT in Mammalian Cells: Inducible and Repressible Expression of hTERT**

The invention provides vectors that can be manipulated to induce or

repress the expression of the TRTs of the invention, such as hTRT. For example, the hTRT coding sequence can be cloned into the Ecdysone-Inducible Expression System from Invitrogen (San Diego, CA) and the Tet-On and Tet-off tetracycline regulated systems from Clontech Laboratories, Inc. (Palo Alto, CA). Such inducible expression systems are provided for use in the methods of the invention where it is important to control the level or rate of transcription of transfected TRT. For example, the invention provides for cell lines immortalized through the expression of hTRT; such cells can be rendered "mortal" by inhibition of hTRT expression by the vector through transcriptional controls, such as those provided by the Tet-Off system. The invention also provides for methods of expressing TRT only transiently to avoid the constitutive expression of hTRT, which may lead to unwanted "immortalization" of the transfected cells, as discussed above.

The Ecdysone-Inducible Mammalian Expression System is designed to allow regulated expression of the gene of interest in mammalian cells. The system is distinguished by its tightly regulated mechanism that allows almost no detectable basal expression and greater than 200-fold inducibility in mammalian cells. The expression system is based on the heterodimeric ecdysone receptor of *Drosophila*. The Ecdysone-Inducible Expression System uses a steroid hormone ecdysone analog, muristerone A, to activate expression of hTRT via a heterodimeric nuclear receptor. Expression levels have been reported to exceed 200-fold over basal levels with no effect on mammalian cell physiology "Ecdysone-Inducible Gene Expression in Mammalian Cells and Transgenic Mice" (1996) *Proc. Natl. Acad. Sci. USA* 93, 3346-3351). Once the receptor binds ecdysone or muristerone, an analog of ecdysone, the receptor activates an ecdysone-responsive promoter to give controlled expression of the gene of interest. In the Ecdysone-Inducible Mammalian Expression System, both monomers of the heterodimeric receptor are constitutively expressed from the same vector, pVgRXR. The ecdysone-responsive promoter, which ultimately drives expression of the gene of interest, is located on a second vector, pIND, which drives the transcription of the gene of interest.

The hTRT coding sequence is cloned in the pIND vector (Clontech Laboratories, Inc, Palo Alto, CA), which contains 5 modified ecdysone response

elements (E/GREs) upstream of a minimal heat shock promoter and the multiple cloning site. The construct is then transfected in cell lines which have been pre-engineered to stably express the ecdysone receptor. After transfection, cells are treated with muristerone A to induce intracellular expression from pIND.

5 The Tet-on and Tet-off expression systems (Clontech, Palo Alto, CA) give access to the regulated, high-level gene expression systems described by Gossen (1992) "Tight control of gene expression in mammalian cells by tetracycline responsive promoters" *Proc. Natl. Acad. Sci. USA* 89:5547-5551, for the Tet-Off transcription repression system; and Gossen (1995) "Transcriptional activation by tetracycline in
10 mammalian cells" *Science* 268:1766-1769, for the Tet-On inducible transcriptional system. In "Tet-Off" transformed cell lines, gene expression is turned on when tetracycline (Tc) or doxycycline ("Dox;" a Tc derivative) is removed from the culture medium. In contrast, expression is turned on in Tet-On cell lines by the addition of Tc or Dox to the medium. Both systems permit expression of cloned genes to be regulated
15 closely in response to varying concentrations of Tc or Dox.

 This system uses the "pTRE" as a response plasmid that can be used to express a gene of interest. Plasmid pTRE contains a multiple cloning site (MCS) immediately downstream of the Tet-responsive PhCMV*-I promoter. Genes or cDNAs of interest inserted into one of the sites in the MCS will be responsive to the
20 tTA and rTA regulatory proteins in the Tet-Off and Tet-On systems, respectively. PhCMV*-I contains the Tet-responsive element (TRE), which consists of seven copies of the 42-bp tet operator sequence (tetO). The TRE element is just upstream of the minimal CMV promoter (PminCMV), which lacks the enhancer that is part of the complete CMV promoter in the pTet plasmids. Consequently, PhCMV*-I is silent in
25 the absence of binding of regulatory proteins to the tetO sequences. The cloned insert must have an initiation codon. In some cases, addition of a Kozak consensus ribosome binding site may improve expression levels; however, many cDNAs have been efficiently expressed in Tet systems without the addition of a Kozak sequence. pTRE-Gene X plasmids are cotransfected with pTK-Hyg to permit selection of stable
30 transfectants.

 Setting up a Tet-Off or Tet-On expression system generally requires two

consecutive stable transfections to create a "double-stable" cell line that contains integrated copies of genes encoding the appropriate regulatory protein and TRT under the control of a TRE. In the first transfection, the appropriate regulatory protein is introduced into the cell line of choice by transfection of a "regulator plasmid" such as pTet-Off or pTet-On vector, which expresses the appropriate regulatory proteins. The hTERT cloned in the pTRE "response plasmid" is then introduced in the second transfection to create the double-stable Tet-Off or Tet-On cell line. Both systems give very tight on/off control of gene expression, regulated dose-dependent induction, and high absolute levels of gene expression.

Expression Recombinant hTERT With DHFR and Adenovirus Sequences

The pGRN155 plasmid construct was designed for transient expression of hTERT cDNA in mammalian cells. A Kozak consensus is inserted at the 5' end of the hTERT sequence. The hTERT insert contains no 3' or 5' UTR. The hTERT cDNA is inserted into the EcoRI site of p91023(B) (Wong (1985) *Science* 228:810-815). The hTERT insert is in the same orientation as the DHFR ORF. This makes the expression vector particularly useful for transient expression.

Plasmid pGRN155 contains the SV40 origin and enhancer just upstream of an adenovirus promoter, a tetracycline resistance gene, an *E. coli* origin and an adenovirus VAI and VAI gene region. This expression cassette contains, in the following order: the adenovirus major late promoter; the adenovirus tripartite leader; a hybrid intron consisting of a 5' splice site from the first exon of the tripartite leader and a 3' splice site from the mouse immunoglobulin gene; the hTERT cDNA; the mouse DHFR coding sequence; and, the SV40 polyadenylation signal.

The adenovirus tripartite leader and the VA RNAs have been reported to increase the efficiency with which polycistronic mRNAs are translated. DHFR sequences have been reported to enhance the stability of hybrid mRNA. DHFR sequences also can provide a marker for selection and amplification of vector sequences. See Logan (1984) *Proc. Natl. Acad. Sci. USA* 81:3655; Kaufman (1985) *Proc. Natl. Acad. Sci. USA* 82: 689 ; and Kaufman (1988) *Focus* (Life Technologies, Inc.), Vol.10, no. 3).

Other expression plasmids of the invention are described for illustrative purposes.

pGRN121

5 The EcoRI fragment from lambda clone 25-1.1.6 containing the entire cDNA encoding hTERT protein was inserted into the EcoRI site of pBluescriptII SK+ such that the 5' end of the cDNA is near the T7 promoter in the vector. The selectable marker that is used with this vector is ampicillin.

pGRN122

10 The NotI fragment from pGRN121 containing the hTERT ORF was inserted into the NotI site of pEBVHisA so that the coding sequence is operably linked to the RSV promoter. This plasmid expresses a fusion protein composed of a His6 flag fused to the N-terminal of the hTERT protein. The selectable marker that is used with this vector is
15 ampicillin or hygromycin.

pGRN123

The NotI fragment from pGRN121 containing the hTERT ORF was inserted into the NotI site of pEBVHisA so that the coding sequence is in the opposite orientation as the
20 RSV promoter, thus expressing *antisense* hTERT.

pGRN124

Plasmid pGRN121 was deleted of all ApaI sites followed by deletion of the MscI-HincII fragment containing the 3'UTR. The Nco-XbaI fragment containing the
25 stop codon of the hTERT coding sequence was then inserted into the Nco-XbaI sites of pGRN121 to make a plasmid equivalent to pGRN121 except lacking the 3'UTR, which may be preferred for increased expression levels in some cells.

pGRN125

30 The NotI fragment from pGRN124 containing the hTERT coding sequence was inserted into the NotI site of pBBS235 so that the open reading frame is in the opposite

orientation of the Lac promoter. The selectable marker that is used with this vector is chloramphenicol.

pGRN126

- 5 The NotI fragment from pGRN124 containing the hTERT coding sequence was inserted into the NotI site of pBBS235 so that the hTERT coding sequence inserted is in the same orientation as the Lac promoter.

pGRN127

- 10 The oligonucleotide 5'-TGCGCACGTGGGAAGCCCTGGCagatctgAattCCaCcATGC CGCGCGCTCCCCGCTG-3' was used *in vitro* mutagenesis of pGRN125 to convert the initiating ATG codon of the hTERT coding sequence into a Kozak consensus sequence and create EcoRI and BglII sites for cloning. Also, oligonucleotide COD2866 was used to convert AmpS to AmpR (ampicillin resistant) and
15 oligonucleotide COD1941 was used to convert CatR (chloramphenicol resistant) to CatS (chloramphenicol sensitive).

pGRN128

- The oligonucleotide 5'-TGCGCACGTGGGAAGCCCTGGCagatctgAattCCaCcATG
20 CCGCGCGCTCCCCGCTG-3' was used *in vitro* mutagenesis to convert the initiating ATG codon of hTERT into a Kozak consensus and create EcoRI and BglII sites for cloning. Also, oligo 5'-CTGCCCTCAGACTTCAAGACCATCCTGGACTACAA GGACGACGATGACAAATGAATTCAGATCTGCGGCCGCCACCGCGGTGGAG CTCCAGC-3' was used to insert the IBI Flag (International Biotechnologies Inc. (IBI),
25 Kodak, New Haven, CT) at the C-terminus and create EcoRI and BglII sites for cloning. Also, COD2866 was used to convert AmpS to AmpR and COD1941 was used to convert CatR to CatS.

pGRN129

- 30 The oligonucleotide 5'-CGGGACGGGCTGCTCCTGCGTTTGGTGGAcGcgTTCTTG TTGGTGACACCTCACCTCACC-3' was used by *in vitro* mutagenesis to convert

Asp869 to an Ala codon (i.e. the second Asp of the DD motif was converted to an Alanine to create a dominant/negative hTERT mutant). This also created a MluI site. Also, oligonucleotide 5'-CTGCCCTCAGACTTCAAGACCATCCTGGACTACAAGGACGACGATGACAAATGAATTCAGATCTGCGGCCGCCACCGCGGTGGAGCTC
 5 CAGC-3') was used to insert the IBI Flag at the C-terminus and create EcoRI and BglII sites for cloning. Also, COD2866 was used to convert AmpS to AmpR and COD1941 was used to convert CatR to CatS.

pGRN130

10 The oligonucleotide 5'-CGGGACGGGCTGCTCCTGCGTTTGGTGGAcGcgTTCTTGTTGGTGACACCTCACCTCACC-3' was used in *in vitro* mutagenesis to convert the Asp869 codon into an Ala codon (i.e. the second Asp of the DD motif was converted to an Alanine to make a dominant/negative variant protein). This also created an MluI site. Also, the oligonucleotide 5'-TGCGCACGTGGGAAGCCCTGGCagatctgAatt
 15 CCaCcATGCCGCGCGCTCCCCGCTG-3' was used in *in vitro* mutagenesis to convert the initiating ATG codon of the hTERT coding sequence into a Kozak consensus sequence and create EcoRI and BglII sites for cloning. Also, COD2866 was used to convert AmpS to AmpR and COD1941 was used to convert CatR.

20 pGRN131

The EcoRI fragment from pGRN128 containing the hTERT ORF with Kozak sequence and IBI Flag mutations is inserted into the EcoRI site of pBBS212 so that the hTERT ORF is expressed off the MPSV promoter. Plasmid pBBS212 contains a MPSV promoter, the CMV enhancer, and the SV40 polyadenylation site.

25 pGRN132

The EcoRI fragment from pGRN128 containing the hTERT ORF with Kozak sequence and IBI Flag mutations is inserted into the EcoRI site of pBBS212 so that the antisense of the hTERT ORF is expressed off the MPSV promoter.

30 pGRN133

The EcoRI fragment from pGRN121 containing the hTRT coding sequence was inserted into the EcoRI site of pBBS212 so that the hTRT protein is expressed under the control of the MPSV promoter.

5 **pGRN134**

The EcoRI fragment from pGRN121 containing the hTRT coding sequence was inserted into the EcoRI site of pBBS212 so that the *antisense* of the hTRT coding sequence is expressed under the control of the MPSV promoter. The selectable markers used with this vector are Chlor/HygB/PAC.

10

pGRN135

Plasmid pGRN126 was digested to completion with MscI and SmaI and religated to delete over 95% of the hTRT coding sequence inserted. One SmaI-MscI fragment was re-inserted during the process to recreate the Cat activity for selection. This unpurified
15 plasmid was then redigested with Sall and EcoRI and the fragment containing the initiating codon of the hTRT coding sequence was inserted into the Sall-EcoRI sites of pBBS212. This makes an *antisense* expression plasmid expressing the antisense of the 5'UTR and 73 bases of the coding sequence. The selectable markers used with this vector are Chlor/HygB/PAC.

20

pGRN136

The HindIII-Sall fragment from pGRN126 containing the hTRT coding sequence was inserted into the HindIII-Sall sites of pBBS242.

25 **pGRN137**

The Sall-Sse8387I fragment from pGRN130 containing the Kozak sequence was inserted into the Sall-Sse8387I sites of pGRN136.

pGRN138

30 The EcoRI fragment from pGRN124 containing hTRT minus the 3'UTR was inserted into the EcoRI site of pEGFP-C2 such that the orientation of the hTRT is the same as

the EGFP domain.

pGRN139

The oligonucleotide 5'- CTGCCCTCAGACTTCAAGACCATCCTGGACTACAAGG
5 ACGACGATGACAAATGAATTCAGATCTGCGGCCGCCACCGCGGTGGAGCTC

CAGC-3 was used in in vitro mutagenesis to insert the IBI Flag at the C-terminus of hTERT in pGRN125 and create EcoRI and BglII sites for cloning. Also, COD2866 was used to convert AmpS to AmpR) and COD1941 was used to convert CatR to CatS.

10 **pGRN140**

The NcoI fragment containing the upstream sequences of genomic hTERT and the first intron of hTERT from lambdaG55 was inserted into the NcoI site of pBBS167. The fragment is oriented so that hTERT is in the same direction as the Lac promoter.

15 **pGRN141**

The NcoI fragment containing the upstream sequences of genomic hTERT and the first intron of hTERT from lambdaG55 was inserted into the NcoI site of pBBS167. The fragment is oriented so that hTERT is in the opposite direction as the Lac promoter.

pGRN142

20 The NotI fragment from lambdaGphi5 containing the complete ~15 kbp genomic insert including the hTERT gene promoter region was inserted in the NotI site of plasmid pBBS185. The fragment is oriented so that the hTERT ORF is in the opposite orientation as the Lac promoter.

25 **pGRN143**

The NotI fragment from lambdaGphi5 containing the complete ~15 kbp genomic insert including the hTERT gene promoter region was inserted in the NotI site of plasmid pBBS185. The fragment is oriented so that the hTERT ORF is in the same orientation as the Lac promoter.

30 **pGRN144**

SAL1 deletion of pGRN140 to remove lambda sequences.

pGRN145

This vector is an intermediate vector for constructing a hTRT fusion protein expression vector. The EcoRI fragment from pGRN137 containing the hTRT coding sequence was inserted into the EcoRI site of pBBS212 to remove the portion of the sequence corresponding to the 5'UTR of hTRT mRNA. The hTRT coding sequence is oriented so that it is expressed under the control of the MPSV promoter. The selectable markers used with this vector are Chlor/HygB/PAC.

pGRN146

- 10 This vector was constructed for the expression of hTRT sequences in mammalian cells. The Sse8387I-NotI fragment from pGRN130 containing the D869A mutation of hTRT was inserted into the Sse8387I-NotI sites of pGRN137. The selectable markers used with this vector are Ampicillin/HygB/PAC.

15 pGRN147

The Sse8387I-NotI fragment from pGRN139 containing the IBI Flag was inserted into the Sse8387I-NotI sites of pGRN137.

pGRN148

- 20 The BglII-Eco47III fragment from pGRN144 containing the promoter region of hTRT was inserted into the BglII-NruI sites of pSEAP2 to make an hTRT promoter/reporter construct.

pGRN149

- 25 This vector was constructed for the expression and mutagenesis of TRT sequences in *E. coli*. The mutagenic oligo 5'-cttcaagaccatcctggactttcgaaacgcggccgccaccgcggtagctcc-3' was used to add a CSP45I site at the C-terminus of hTRT by *in vitro* mutagenesis of pGRN125. The "stop" codon of hTRT was deleted and replaced with a Csp45I site. The selectable marker that is used with this vector is ampicillin.

30

pGRN150

The BglII-FspI fragment from pGRN144 containing the promoter region of hTRT was inserted into the BglII-NruI sites of pSEAP2 to make an hTRT promoter/reporter construct.

5 pGRN151

This vector was constructed for the expression of hTRT sequences in mammalian cells. The EcoRI fragment from pGRN147 containing the hTRT coding sequence was inserted into the EcoRI site of pBBS212 to remove the portion of the sequence corresponding to the 5'UTR of the hTRT mRNA. The hTRT coding sequence is oriented so that it is expressed under the control of the MPSV promoter. The selectable markers used with this vector are Chlor/HygB/PAC.

pGRN152

The EcoRI fragment from pGRN146 containing the hTRT coding sequence was inserted into the EcoRI site of pBBS212 to remove the portion of the sequence corresponding to the 5'UTR of the hTRT. The hTRT coding sequence is oriented so that it is expressed under the control of the MPSV promoter.

pGRN153

The StyI fragment from pGRN130 containing the D869-->A mutation of hTRT (hTRT variant coding sequence) was inserted into the StyI sites of pGRN158 to make a plasmid containing the hTRT coding sequence with a Kozak consensus sequence at its 5'-end, an IBI FLAG sequence at its 3'-end (the C-terminus encoding region), and the D869-->A mutation.

pGRN154

The EcoRI fragment of pGRN153 containing the hTRT gene was inserted into the EcoRI site of plasmid pBS212 in an orientation such that the hTRT ORF is oriented in the same direction as the MPSV promoter. This makes an MPSV-directed expression plasmid that expresses the hTRT protein with a Kozak consensus sequence at its amino-terminal end, an IBI FLAG at its carboxy-terminal end, and the D869-->A mutation.

pGRN155

This vector was constructed for the expression of hTRT sequences in mammalian cells. The insert included full length cDNA of hTRT minus 5' and 3' UTR, and Kozak sequences. The EcoRI fragment from pGRN145 containing the hTRT cDNA with the

Kozak consensus and no 3' or 5' UTR was inserted into the EcoRI site of p91023(B) such that the hTERT is in the same orientation as the DHFR ORF. This makes a transient expression vector for hTERT. The selectable marker used with this vector is tetracycline.

5

pGRN156

This vector was constructed for the expression of hTERT sequences in mammalian cells. The EcoRI fragment from pGRN146 containing the D869A mutation of the hTERT cDNA with the Kozak consensus and no 3' or 5' UTR was inserted into the EcoRI site of p91023(B) such that the hTERT is in the same orientation as the DHFR ORF. This makes a transient expression vector for hTERT. The insert included full length cDNA of hTERT minus 5' and 3' UTR, D869A, and Kozak sequences. The selectable marker used with this vector is tetracycline.

10

pGRN157

This vector was constructed for the expression of hTERT sequences in mammalian cells. The EcoRI fragment from pGRN147 containing the hTERT cDNA with the IBI FLAG at the C-terminus; the Kozak consensus and no 3' or 5' UTR into the EcoRI site of p91023(B) such that the hTERT is in the same orientation as the DHFR ORF. This makes a transient expression vector for hTERT. The insert included full length cDNA of hTERT minus 5' and 3' UTR, the FLAG sequence, and Kozak sequences. The selectable marker used with this vector is tetracycline.

20

pGRN158

This vector was constructed for the expression and mutagenesis of TRT sequences in *E. coli*. The EcoRI fragment from pGRN151 containing the hTERT ORF was inserted into the EcoRI site of pBBS183 so that the hTERT ORF is oriented in the opposite direction as the Lac promoter. The insert included full length cDNA of hTERT minus 5' and 3' UTR, FLAG sequence, and Kozak sequences. The hTERT coding sequence is driven by a T7 promoter. The selectable marker used with this vector is ampicillin.

30

pGRN159

This vector was constructed for the expression and mutagenesis of TRT sequences in *E. coli*. The *HeI*-*KpnI* fragment from pGRN138 containing the EGFP to hTRT fusion was inserted into the *XbaI*-*KpnI* sites of pBluescriptIIKS+. This makes a T7 expression
5 vector for the fusion protein (the coding sequence is driven by a T7 promoter). The insert included full length cDNA of hTRT minus the 3' UTR as a fusion protein with EGFP. The selectable marker used with this vector is ampicillin.

pGRN160

10 This vector was constructed for the expression of *antisense* hTR sequences in mammalian cells. The coding sequence is operably linked to an MPSV promoter. The *XhoI*-*NsiI* fragment from pGRN90 containing the full length hTR ORF was inserted into the *Sall*-*Sse8387I* sites of pBBS295. This makes a transient/stable vector
15 expressing hTR antisense RNA. A GPT marker was incorporated into the vector. The selectable markers used with this vector are Chlor/gpt/PAC.

pGRN161

This vector was constructed for the expression of *sense* hTR sequences in mammalian cells. The *XhoI*-*NsiI* fragment from pGRN89 containing the full length hTR ORF was
20 inserted into the *Sall*-*Sse8387I* sites of pBBS295. This makes a transient/stable vector expressing hTR in the sense orientation. The coding sequence is driven by an MPSV promoter. A GPT marker was incorporated into the vector. The selectable markers used with this vector are Chlor/gpt/PAC.

25 pGRN162

The *XhoI*-*NsiI* fragment from pGRN87 containing the full length hTR ORF was inserted into the *Sall*-*Sse8387I* sites of pBBS295. This makes a transient/stable vector
expressing truncated hTR (from position +108 to +435) in the sense orientation.

30 pGRN163

This vector was constructed for the expression and mutagenesis of TRT sequences in *E.*

coli. The coding sequence is driven by a T7 promoter. Oligonucleotide RA45 (5'-GCCACCCCCGCGCTGCCTCGAGCTCCCCGCTGC-3') is used

XhoI site in the next two codons after the Leu. Also COD 1941 was used to change CatR to CatS, and introduces a BSPHI site, and COD 2866 was used to change AmpS to AmpR, introducing an FSP1 site. The selectable marker used with this vector is ampicillin.

pGRN164

This vector was constructed for the expression of hTR sequences in *E. coli*. Primers hTR +1 5'-GGGGAAGCTTTAATACGACTCACTATAGGGTTGCGGAGGGTGG GCCTG-3' and hTR +445 5'-CCCCGGATCCTGCGCATGTGTGAGCCGAGTCCT GGG-3' were used to amplify by PCR a fragment from pGRN33 containing the full length hTR with the T7 promoter on the 5' end (as in hTR+1). A BamHI-HindIII digest of the PCR product was put into the BamHI-HindIII sites of pUC119. The coding sequence operably linked to a T7 promoter. The selectable marker used with this vector is ampicillin.

pGRN165

This vector was constructed for the expression and mutagenesis of hTRT sequences in *E. coli*. The coding sequence is operably linked to a T7 promoter. The EcoRI fragment from pGRN145 containing the hTRT ORF with a Kozak front end was inserted into the EcoRI site of pBluescriptIIISK+ so that the hTRT is oriented in the same direction as the T7 promoter. The selectable marker used with this vector is ampicillin.

25 pGRN166

This vector was constructed for the expression and mutagenesis of TRT sequences in mammalian cells. The coding sequence is operably linked to a T7 promoter. The EcoRI fragment from pGRN151 containing the hTRT ORF with a Kozak front end and IBI flag at the back end was inserted into the EcoRI site of pBluescriptIIISK+ so that the hTRT ORF is oriented in the same direction as the T7 promoter. The insert included full length cDNA of hTRT minus 5' and 3' UTR. FLAG sequence (Immunex Corp.

Seattle WA), and Kozak sequences. The selectable marker used with this vector is ampicillin.

pGRN167

5 AvRII-StuI fragment from pGRN144 containing the 5' end of the hTERT ORF was inserted into the XbaI-StuI sites of pBBS161.

pGRN168

10 The EcoRI fragment from pGRN145 containing the optimized hTERT expression cassette was inserted into the EcoRI site of pIND such that the hTERT coding sequence is in the same orientation as the miniCMV promoter.

pGRN169

15 The EcoRI fragment from pGRN145 containing the optimized hTERT expression cassette was inserted into the EcoRI site of pIND such that the hTERT is in the reverse orientation from the miniCMV promoter.

pGRN170

20 The EcoRI fragment from pGRN145 containing the optimized hTERT expression cassette was inserted into the EcoRI site of pIND(spl) such that the hTERT is in the opposite orientation from the miniCMV promoter.

25

30

amphicillin/neomycin/kanamycin.

pGRN171

5 The Eco47III-NarI fragment from pGRN163 was inserted into the Eco47III-NarI sites of pGRN167, putting the M1L mutation into a fragment of the hTERT genomic DNA.

pGRN172

10 The BamHI-StuI fragment from pGRN171 containing the Met to Leu mutation in the hTERT ORF was inserted into the BglII-NruI sites of pSEAP2-Basic.

pGRN173

15 The EcoRV-Eco47III fragment from pGRN144 containing the 5' end of the hTERT promoter region was inserted into the SrfI-Eco47III sites of pGRN172. This makes a promoter reported plasmid that contains the promoter region of hTERT from approximately 2.3 kb upstream from the start of the hTERT ORF to just after the first intron in the coding region, with the Met1-->Leu mutation.

pGRN174

20 The EcoRI fragment from pGRN145 containing the "optimized" hTERT expression cassette was inserted into the EcoRI site of pIND(sp1) such that the hTERT is in the same orientation as the miniCMV promoter.

25

EXAMPLE 7

RECONSTITUTION OF TELOMERASE ACTIVITY

A. Co-Expression of hTERT and hTR *in vitro*

In this example, the coexpression of hTERT and hTR using an *in vitro* cell-free expression system is described. These results demonstrate that the hTERT polypeptide encoded by pGRN121 encodes a catalytically active telomerase protein and that *in vitro* reconstitution (IVR) of the telomerase RNP can be accomplished using recombinantly expressed hTERT and hTR.

Telomerase activity was reconstituted by adding linearized plasmids of hTERT (pGRN121; 1 µg DNA digested with Xba I) and hTR (phTR+1; 1 µg digested with FspI) to a coupled transcription-translation reticulocyte lysate system (Promega TNTTM). phTR+1 is a plasmid which, when linearized with FspI and then transcribed by T7 RNA polymerase, generates a 445 nucleotide transcript beginning with nucleotide +1 and extending to nucleotide 446 of hTR (Autexier et al., 1996, *EMBO J* 15:5928). For a 50 µl reaction the following components were added: 2 µl TNTTM buffer, 1 µl TNTTM T7 RNA polymerase, 1 µl 1 mM amino acid mixture, 40 units RnasinTM RNase inhibitor, 1 µg each linearized template DNA, and 25 µl TNTTM reticulocyte lysate. Components were added in the ratio recommended by the manufacturer and were incubated for 90 min at 30°C. Transcription was under the direction of the T7 promoter and could also be carried out prior to the addition of reticulocyte lysate with similar results. After incubation, 5 and 10 µl of the programmed transcription-translation reaction were assayed for telomerase activity by TRAP as previously described (Autexier et al., *supra*) using 20 cycles of PCR to amplify the signal.

The results of the reconstitution are shown in Figure 10. For each transcription/translation reaction assayed there are 3 lanes: The first 2 lanes are duplicate assays and the third lane is a duplicate sample heat denatured (95°C, 5 min) prior to the TRAP phase to rule out PCR generated artifacts.

As shown in Figure 10, reticulocyte lysate alone has no detectable telomerase activity (lane 6). Similarly, no detectable activity is observed when either hTR alone (lane 1) or full length hTERT gene (lane 4) are added to the lysate. When

both components are added (lane 2), telomerase activity is generated as demonstrated by the characteristic repeat ladder pattern. When the carboxyl-terminal region of the hTERT gene is removed by digestion of the vector with *NcoI* ("truncated hTERT") telomerase activity is abolished (lane 3). Lane 5 shows that translation of the truncated hTERT alone does not generate telomerase activity. Lane "R8" shows a positive control for a telomerase product ladder generated by TRAP of TSR8, a synthetic telomerase product having a nucleotide sequence of 5'-ATTCCGTCGAGCAGAGTTAG[GGTTAG]₇-3'.

10 B. Mixing of hTERT and hTR *in vitro*

In vitro reconstitution of telomerase activity was also accomplished by mixing. hTERT was transcribed and translated as described *supra*, but without the addition of the hTR plasmid. Reconstitution of the telomerase RNP was then accomplished by mixing the hTERT translation mixture with hTR (previously generated by T7 RNA polymerase transcription from phTR+1-Fsp) in the ratio of 2 µl of hTERT translation mix to 2 µl of hTR (1 µg) then incubated for 90 minutes at 30° C. This method of hTERT:hTR reconstitution is referred to as "linked reconstitution" or "linked IVR." Telomerase activity is present (i.e., can be detected) in this mixture. Improved signal was observed following partial purification of the activity by DEAE chromatography. In this case Millipore Ultrafree-MC DEAE Centrifugal Filter Devices were used according to the manufacturer's directions). The buffers used were hypo0.1, hypo0.2, and hypo1.0, where hypo is 20 mM Hepes-KOH, pH 7.9, 2 mM MgCl₂, 1 mM EGTA, 10 % glycerol, 0.1 % NP-40, 1 mM DTT, 1 mM Na-metabisulfite, 1 mM benzamidine, and 0.2 mM phenylmethylsulfonylfluoride (PMSF), and where 0.1, 0.2 and 1.0 refers to 0.1, 0.2 or 1.0 M KCL. The filters were pre-conditioned with hypo1.0, washed with hypo0.1, the reconstituted telomerase was loaded, the column was washed with hypo0.1 then hypo0.2, and the reconstituted telomerase was eluted with hypo1.0 at half the volume as was loaded. This formulation could be stored frozen at -70°C and retains activity.

30 Telomerase activity was assayed in a two step procedure. In step one, a conventional telomerase assay was performed as described in Morin, 1989, *Cell* 59:

521, except no radiolabel was used. In step two, an aliquot was assayed by the TRAP procedure for 20-30 cycles as described *supra*. The conventional assay was performed by assaying 1-10 μ l of reconstituted telomerase in 40-50 μ l final volume of 25 mM Tris-HCl, pH 8.3, 50 mM K-acetate, 1 mM EGTA, 1 mM MgCl₂, 2 mM dATP, 2 mM TTP, 10 μ M dGTP, and 1 μ M primer (usually M2, 5'-AATCCGTCGAGCAGAGTT) at 30° C for 60-180 minutes. The reaction was stopped by heating to 95° C for 5 minutes and 1-10 μ l of the first step mixture was carried onto the step two TRAP reaction (50 μ l).

In additional experiments, the synthesis of hTRT and hTR during *in vitro* reconstitution was monitored by ³⁵S-methionine incorporation and Northern blotting, respectively. Proteins of approximately the predicted size were synthesized for hTRT (127 kD), hTRT-Nco (85 kD), and pro90hTRT (90 kD) in approximately equal molar amounts relative to each other. The Northern analysis indicated hTR synthesis was the correct size (445 nucleotides) and predominantly intact.

Variations of the reconstitution protocols, *supra*, will be apparent to those of skill. For example, the time and temperature of reconstitution, and presence or concentration of components such as monovalent salt (e.g., NaCl, KCl, potassium acetate, potassium glutamate, and the like), divalent salt (MgCl₂, MnCl₂, MgSO₄, and the like), denaturants (urea, formamide, and the like), detergents (NP-40, Tween, CHAPS, and the like), and alternative improved purification procedures (such as immunoprecipitation, affinity or standard chromatography) can be employed. These and other parameters can be varied in a systematic way to optimize conditions for particular assays or other reconstitution protocols.

C. Reconstitution Using hTRT Variants and Fusion Proteins

Reconstitution of telomerase catalytic activity occurred when EGFP-hTRT, a fusion of the enhanced green fluorescent protein to hTRT (see Examples 6 and 15), or epitope-tagged hTRT (IBI FLAG, see Example 6) both reconstituted telomerase activity at approximately wild-type levels were coexpressed with hTR.

In contrast, telomerase activity was not reconstituted when a variant

hTERT, pro90hTERT (missing RT motifs B', C, D, and E) was used. This demonstrates that pro90hTERT does not possess full telomerase catalytic activity, although it may have other partial activities (e.g., RNA [i.e. hTR] binding ability and function as dominant-negative regulator of telomerase *in vivo* as described *supra*).

5

D. Assay of *in vitro* Reconstituted Telomerase Activity Using the Gel Blot and Conventional Telomerase Assay

The following example demonstrates that *in vitro* reconstituted (IVR) telomerase can be assayed using conventional telomerase assays in addition to amplification-based assays (i.e., TRAP). IVR telomerase as described in part (B), *supra* (the "linked reconstitution method") followed by DEAE purification, as described *supra* was assayed using the gel blot assay using the following reaction conditions; 1-10 μ l of linked IVR telomerase in 40 μ l final volume of 25 mM Tris-HCl, pH 8.3, 50 mM K-acetate, 1 mM EGTA, 1 mM MgCl₂, 0.8 mM dATP, 0.8 mM TTP, 1.0 mM dGTP, and 1 μ M primer (M2, *supra*; or H3.03, 5'-TTAGGGTTAGGGTTAGGG) at 30°C for 180 minutes. The telomeric DNA synthesized was isolated by standard procedures, separated on a 8 % polyacrylamide, 8 M urea gel, transferred to a nylon membrane, and probed using the ³²P-(CCCTAA)_n riboprobe used in the dot-blot assay. The probe identified a six nucleotide ladder in the lane representing 10 μ l of IVR telomerase that was equivalent to the ladder observed for 5 μ l of native nuclear telomerase purified by mono Q and heparin chromatography. The results show that IVR telomerase possesses processive telomerase catalytic activity equivalent to native telomerase.

Linked IVR telomerase was also assayed by the conventional ³²P-dGTP incorporation telomerase assay. IVR telomerase prepared by the linked reconstitution method followed by DEAE purification, as described above, was assayed under both processive and non-processive reaction conditions. Assay conditions were 5-10 μ l of linked IVR telomerase in 40 μ l final volume of 25 mM Tris-HCl, pH 8.3, 50 mM K-acetate, 1 mM EGTA, 1 mM MgCl₂, 2 mM dATP, 2 mM TTP, with 10 μ M ³²P-dGTP (72 Ci/mmol) [for assay of processive conditions] or 1 μ M ³²P-dGTP (720 Ci/mmol) [for non-processive], and 1 μ M primer (i.e., H3.03, *supra*) at 30°C [for the

processive reaction] or 37°C [for the non-processive reaction] for 180 minutes. The telomeric DNA synthesized was isolated by standard procedures and separated on a 8 % polyacrylamide, 8 M urea gel sequencing gel. The processive reaction showed a weak six nucleotide ladder consistent with a processive telomerase reaction, and the non-processive reaction added one repeat, a pattern equivalent to a control reaction with a native telomerase preparation. Conventional assays using IVR telomerase are useful in screens for telomerase modulators, as described herein, as well as other uses such as elucidation of the structural and functional properties of telomerase.

10 E. *In vitro* Reconstituted Telomerase Recognizes Primer 3' Termini

This experiment demonstrates that IVR telomerase recognizes primer 3' termini equivalently to native (purified) telomerase. Telomerase forms a base-paired duplex between the primer 3' end and the template region of hTR and adds the next specified nucleotide (Morin, 1989. *supra*). To verify that IVR (recombinant) telomerase has the same property, the reactions of primers with ---GGG or ---TAG 3' termini (AATCCGTCGAGCAGAGGG and AATCCGTCGAGCAGATAG) were compared to a primer having a ---GTT 3' terminus (M2 *supra*) using IVR and native telomerase assayed by the two step conventional/TRAP assay detailed above. The product ladders of the ---GGG and ---TAG primers were shifted +4 and +2, respectively, when compared to the standard primer (---GTT 3' end). The same effect as was observed with native telomerase. This experiment demonstrates IVR and native telomerases recognize primer termini in a similar manner.

These results (along with the results *supra* showing that IVR telomerase possesses both processive and non-processive catalytic activity) indicate that IVR telomerase has similar structure and properties compared to native or purified telomerase.

EXAMPLE 8

PRODUCTION OF ANTI-HTRT ANTIBODIES

30 A. Production of Anti-hTRT Antibodies Against hTRT Peptides

To produce anti-hTRT antibodies, the following peptides from hTRT

were synthesized with the addition of C (cysteine) as the amino terminal residue (see Figure 54).

S-1: FFY VTE TTF QKN RLF FYR KSV WSK

S-2: RQH LKR VQL RDV SEA EVR QHR EA

5 S-3: ART FRR EKR AER LTS RVK ALF SVL NYE

A-3: PAL LTS RLR FIP KPD GLR PIV NMD YVV

The cysteine moiety was used to immobilize (i.e., covalently link) the peptides to BSA and KLH [keyhole limpet hemocyanin] carrier proteins. The KLH-peptides were used as antigen. The BSA-peptide conjugates served as material for ELISAs for testing the
10 specificity of immune antisera.

The KLH-peptide conjugates were injected into New Zealand White rabbits. The initial injections are made by placing the injectant proximal to the axillary and inguinal lymph nodes. Subsequent injections were made intramuscularly. For initial injections, the antigen was emulsified with Freund's complete adjuvant; for
15 subsequent injections, Freund's incomplete adjuvant was used. Rabbits follow a three week boost cycle, in which 50 ml of blood yielding 20-25 ml of serum is taken 10 days after each boost. Antisera against each of the four peptides recognized the hTERT moiety of recombinant hTERT fusion protein (GST-HIS₆-hTERT-fragment 2426 to 3274); see Example 6) on western blots.

20 Using a partially purified telomerase fraction from human 293 cells (approximately 1000-fold purification compared to a crude nuclear extract) that was produced as described in PCT application No. 97/06012 and affinity purified anti-S-2 antibodies, a 130 kd protein doublet could be detected on a western blot. A sensitive chemiluminescence detection method was employed (SuperSignal chemiluminescence
25 substrates, Pierce) but the signal on the blot was weak, suggesting that hTERT is present in low or very low abundance in these immortal cells. The observation of a doublet is consistent with a post-translational modification of hTERT, i.e., phosphorylation or glycosylation.

For affinity purification, the S-2 peptide was immobilized to SulfoLink
30 (Pierce, Rockford IL) through its N-terminal Cysteine residue according to the manufacturer's protocol. First bleed serum from a rabbit immunized with the KLH-S-2

peptide antigen was loaded over a the S-2-SulfoLink and antibodies specifically bound to the S-2 peptide were eluted.

B. Production of Anti-hTERT Antibodies Against hTERT Fusion Proteins

5 GST-hTERT fusion proteins were expressed in *E. coli* as the GST-hTERT fragment #4 (nucleotides 3272-4177) and the GST-HIS8 -hTERT fragment #3 (nucleotides 2426 to 3274) proteins described in Example 6. The fusion proteins were purified as insoluble protein, and the purity of the antigens was assayed by SDS polyacrylamide gels and estimated to be about 75% pure for the GST-hTERT fragment 10 #4 recombinant protein and more than 75% pure for GST-HIS8 -hTERT fragment #3 recombinant protein. Routine methods may be used to obtain these and other fusion proteins at a purity of greater than 90%. These recombinant proteins were used to immunize both rabbits and mice, as described above.

The first and second bleeds from both the mice and rabbits were tested 15 for the presence of anti-hTERT antibodies after removal of anti-GST antibodies using a matrix containing immobilized GST. The antisera were tested for anti-hTERT antibodies by Western blotting using immobilized recombinant GST-hTERT fusion protein, and by immunoprecipitation using partially purified native telomerase enzyme. While no signal was observed in these early bleeds, titers of anti-hTERT antibodies, as expected, 20 increased in subsequent bleeds.

EXAMPLE 9

DETECTION OF AN hTERT MRNA CORRESPONDING TO A182 RNA

VARIANT

25 Poly A⁺ RNA from human testis and the 293 cell line was analyzed for hTERT mRNA using RT-PCR and nested primers. The first primer set was TCP1.1 and TCP1.15; the second primer set was TCP1.14 and BTCP6. Amplification from each gave two products differing by 182 bp; the larger and smaller products from testis RNA were sequenced and found to correspond exactly to pGRN121(Figure 16) and the 30 712562 clone (Figure 18), respectively. The variant hTERT RNA product has been observed in mRNA from SW39i, OVCAR4, 293, and Testes.

Additional experiments were carried out to demonstrate that the $\Delta 182$ cDNA was not an artifact of reverse transcription. Briefly, full-length hTRT RNA (i.e., without the deletion) was produced by *in vitro* transcription of pGRN121 for use as a template for RT-PCR. Separate cDNA synthesis reactions were carried out using
5 Superscript® reverse transcriptase (Bethesda Research Laboratories, Bethesda MD) at 42° or 50°C, and with random-primers or a specific primer. After 15 PCR cycles the longer product was detectable; however, the smaller product (i.e., corresponding to the deletion) was not detectable even after 30 or more cycles. This indicates that the RT-PCR product is not an artifact.

10

EXAMPLE 10

SEQUENCING OF TESTIS hTRT mRNA

The sequence of the testis form of hTRT RNA was determined by direct manual sequencing of DNA fragments generated by PCR from testis cDNA (Marathon
15 Testes cDNA, Clontech, San Diego CA) using a ThermoSequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science). The PCR step was performed by a nested PCR, as shown in Table 8. In all cases a negative control reaction with primers but no cDNA was performed. The absence of product in the control reaction demonstrated that the products derived from the reaction with cDNA
20 present were not due to contamination of hTRT from pGRN121 or other cell sources (e.g., 293 cells). The DNA fragments were excised from agarose gels to purify the DNA prior to sequencing.

The testis mRNA sequence corresponding to bases 27 to 3553 of the pGRN121 insert sequence, and containing the entire hTRT ORF (bases 56 to 3451) was
25 obtained. There were no differences between the testis and the pGRN121 sequences in this region.

TABLE 8

| Fragment | Primer Set 1 | Primer Set 2 | Final Size | Primers For Seq |
|----------|------------------|-----------------|------------|---|
| OA | na | K320/K322 | 208 | K320, K322 |
| A | K320/TCP1.43 | TCP1.40/TCP1.34 | 556 | TCP1.52, TCP1.39, K322, TCP1.40, TCP1.41, TCP1.30, TCP1.34, TCP1.49 |
| B | TCP1.42/TCP1.32B | TCP1.35/TCP1.21 | 492 | TCP1.35, TCP1.28, TCP1.38, TCP1.21, TCP1.46, TCP1.33, TCP1.48 |
| C | TCP1.65/TCP1.66 | TCP1.67/TCP1.68 | 818 | TCP1.67, TCP1.32, TCP1.69, TCP1.68, TCP1.24, TCP1.44, K303 |
| D2 | K304/bHITCP6 | LT1/TCP1.6 | 546 | LT2, LT1, TCP1.6, bTCP4, TCP1.13, TCP1.77, TCP1.1 |
| D3 | TCP1.12/TCP1.7 | TCP1.14/TCP1.15 | 604 | TCP1.6, TCP1.14, TCP1.73, TCP1.78, TCP1.25, TCP1.15, TCP1.76 |
| EF | na | TCP1.74/TCP1.7 | 201 | TCP1.74, TCP1.7, TCP1.75, TCP1.15, TCP1.3 |
| E | TCP1.3/TCP1.4 | TCP1.2/TCP1.9 | 687 | TCP1.2, TCP1.8, TCP1.9, TCP1.26 |
| F | TCP1.26/UTR2 | TCP1.10/TCP1.4 | 377 | TCP1.4, TCP1.10, TCP1.11 |

EXAMPLE 11

DETECTION OF hTERT mRNA BY RNASE PROTECTION

RNase protection assays can be used to detect, monitor, or diagnose the presence of an hTERT mRNA or variant mRNA. One illustrative RNase protection probe is an *in vitro* synthesized RNA comprised of sequences complementary to hTERT mRNA sequences and additional, non-complementary sequences. The latter sequences are included to distinguish the full-length probe from the fragment of the probe that results from a positive result in the assay: in a positive assay, the complementary sequences of the probe are protected from RNase digestion, because they are hybridized to hTERT mRNA. The non-complementary sequences are digested away from the probe in the presence of RNase and target complementary nucleic acid.

Two RNase protection probes are described for illustrative purposes; either can be used in the assay. The probes differ in their sequences complementary to hTERT, but contain identical non-complementary sequences, in this embodiment, derived from the SV40 late mRNA leader sequence. From 5'-3', one probe is comprised of 33 nucleotides of non-complementary sequence and 194 nucleotides of sequence complementary to hTERT nucleotides 2513 - 2707 for a full length probe size of 227 nucleotides. From 5'-3', the second probe is comprised of 33 nucleotides of non-complementary sequence and 198 nucleotides of sequence complementary to hTERT nucleotides 2837 - 3035 for a full length probe size of 231 nucleotides. To conduct the assay, either probe can be hybridized to RNA, i.e., polyA⁺ RNA, from a test sample, and T1 ribonuclease and RNase A are then added. After digestion, probe RNA is purified and analyzed by gel electrophoresis. Detection of a 194 nucleotide fragment of the 227 nucleotide probe or a 198 nucleotide fragment of the 231 nucleotide probe is indicative of hTERT mRNA in the sample.

The illustrative RNase protection probes described in this example can be generated by *in vitro* transcription using T7 RNA polymerase. Radioactive or otherwise labeled ribonucleotides can be included for synthesis of labeled probes. The templates for the *in vitro* transcription reaction to produce the RNA probes are PCR products. These illustrative probes can be synthesized using T7 polymerase following PCR amplification of pGRN121 DNA using primers that span the corresponding

complementary region of the hTRT gene or mRNA. In addition, the downstream primer contains T7 RNA polymerase promoter sequences and the non-complementary sequences.

For generation of the first RNase protection probe, the PCR product from the following primer pair (T701 and reverse01) is used:

T701 5'-GGGAGATCT TAATACGACTCACTATAG ATTCA GGCCATGGTG CTGCGCCGGC TGTC A GGCTCCC ACGACGTAGT CCATGTTAC-3'; and reverse01 5'-GGGTCTAGAT CCGGAAGAGTGT CTGGAGCAAG-3'.

For generation of the second RNase protection probe, the PCR product from the following primer pair (T702 and reverse02) is used:

T702 5'-GGGAGATCT TAATACGACTCACTATAG ATTCA GGCCATGGTG CTGCGCCGGC TGTC A GGGCG GCCTTCTGGA CCACGGCATA CC-3'; and reverse02 5'-G GTCTAGA CGATATCC ACAGGGCCTG GCGC-3'.

EXAMPLE 12

CONSTRUCTION OF A PHYLOGENETIC TREE COMPARING HTRT AND OTHER REVERSE TRANSCRIPTASES

A phylogenetic tree (Figure 6) was constructed by comparison of the seven RT domains defined by Xiong and Eickbush (1990, *EMBO J.* 9:3353). After sequence alignment of motifs 1, 2, and A-E from 4 TRTs, 67 RTs, and 3 RNA polymerases, the tree was constructed using the NJ (Neighbor Joining) method (Saitou and Nei, 1987, *Mol. Biol. Evol.* 4:406). Elements from the same class that are located on the same branch of the tree are simplified as a box. The length of each box corresponds to the most divergent element within that box.

The TRTs appear to be more closely related to RTs associated with msDNA, group II introns, and non-LTR (Long Terminal Repeat) retrotransposons than to the LTR-retrotransposon and viral RTs. The relationship of the telomerase RTs to the non-LTR branch of retroelements is intriguing, given that these latter elements have replaced telomerase for telomere maintenance in *Drosophila*. However, the most striking finding is that the TRTs form a discrete subgroup, almost as closely related to the RNA-dependent RNA polymerases of plus-stranded RNA viruses such as

poliovirus as to any of the previously known RTs. Considering that the four telomerase genes come from evolutionarily distant organisms -- protozoan, fungi, and mammal -- this separate grouping cannot be explained by lack of phylogenetic diversity in the data set. Instead, this deep bifurcation suggests that the telomerase RTs are an ancient group, perhaps originating with the first eukaryote.

GenBank protein identification or accession numbers used in the phylogenetic analysis were: msDNAs (94535, 134069, 134074, 134075, 134078), group II introns (483039, 101880, 1332208, 1334433, 1334435, 133345, 1353081), mitochondrial plasmid/RTL (903835, 134084), non-LTR retrotransposons (140023, 84806, 103221, 103353, 134083, 435415, 103015, 1335673, 85020, 141475, 106903, 130402, U0551, 903695, 940390, 2055276, L08889), LTR retrotransposons (74599, 85105, 130582, 99712, 83589, 84126, 479443, 224319, 130398, 130583, 1335652, 173088, 226407, 101042, 1078824), hepadnaviruses (I 18876, 1706510, 118894), caulimoviruses (331554, 130600, 130593, 93553), retroviruses (130601, 325465, 74601, 130587, 130671, 130607, 130629, 130589, 130631, 1346746, 130651, 130635, 1780973, 130646). Alignment was analyzed using ClustalW 1.5 [J. D. Thompson, D. G. Higgins, T. J. Gibson, *Nucleic Acids Res* 22, 4673 (1994)] and PHYLIP 3.5 [J. Felsenstein, *Cladistics* 5, 164 (1989)].

EXAMPLE 13

TRANSFECTION OF CULTURED HUMAN FIBROBLASTS (BJ) WITH CONTROL PLASMID AND PLASMID ENCODING hTERT

This example demonstrates that expression of recombinant hTERT protein in a mammalian cell results in the generation of an active telomerase.

Subconfluent BJ fibroblasts were trypsinized and resuspended in fresh medium (DMEM/199 containing 10% Fetal Calf Serum) at a concentration of 4×10^6 cells/ml. The cells were transfected using electroporation with the BioRad Gene Pulser™ electroporator. Optionally, one may also transfect cells using Superfect™ reagent (Qiagen) in accordance with the manufacturer's instructions. For electroporation, 500 μ l of the cell suspension were placed in an electroporation cuvette (BioRad, 0.4 cm electrode gap). Plasmid DNA (2 μ g) was added to the cuvettes and the

suspension was gently mixed and incubated on ice for 5 minutes. The control plasmid (pBBS212) contained no insert behind the MPSV promoter and the experimental plasmid (pGRN133) expressed hTERT from the MPSV promoter. The cells were electroporated at 300 Volts and 960 μ FD. After the pulse was delivered, the cuvettes were placed on ice for approximately 5 minutes prior to plating on 100 mm tissue culture dishes in medium. After 6 hours, the medium was replaced with fresh medium. 72 hours after the transfection, the cells were trypsinized, washed once with PBS, pelleted and stored frozen at -80°C. Cell extracts were prepared at a concentration of 25,000 cells/ μ l by a modified detergent lysis method (see Bodnar et al., 1996, *Exp. Cell Res.* 228:58; Kim et al., 1994, *Science* 266:2011, and as described in patents and publications relating to the TRAP assay, *supra*) and telomerase activity in the cell extracts was determined using a modified PCR-based TRAP assay (Kim et al., 1994, Bodnar et al., 1996). Briefly, 5×10^4 cell equivalents were used in the telomerase primer extension portion of the reaction. While the extract is typically taken directly from the telomerase extension reaction to the PCR amplification, one may also extract once with phenol/chloroform and once with chloroform prior to the PCR amplification. One-fifth of the material was used in the PCR amplification portion of the TRAP reaction (approximately 10,000 cell equivalents). One half of the TRAP reaction was loaded onto the gel for analysis, such that each lane in Figure 25 represents reaction products from 5,000 cell equivalents. Extracts from cells transfected with pGRN133 were positive for telomerase activity while extracts from untransfected (not shown) or control plasmid transfected cells showed no telomerase activity. Similar experiments using RPE cells gave the same result.

Reconstitution in BJ cells was also carried out using other hTERT constructs (i.e., pGRN145, pGRN155 and pGRN138). Reconstitution using these constructs appeared to result in more telomerase activity than in the pGRN133 transfected cells.

The highest level of telomerase activity was achieved using pGRN155. As discussed *supra*, pGRN155 is a vector containing the adenovirus major late promoter as a controlling element for the expression of hTERT and was shown to reconstitute telomerase activity when transfected into BJ cells.

Notably, when reconstitution using the hTERT-GFP fusion protein pGRN138 (which localizes to the nucleus, see Example 15, *infra*) was performed either *in vitro* (see Example 7) or *in vivo* (transfection into BJ cells) telomerase activity resulted. By transfection into BJ cells, for example, as described *supra*, telomerase activity was comparable to that resulting from reconstitution *in vitro* using pGRN133 or pGRN145.

Similar results were obtained upon transfection of normal human retinal pigmented epithelial (RPE) with the hTERT expression vectors of the invention. The senescence of RPE cells is believed to contribute to or cause the disease of age-related macular degeneration. RPE cells treated in accordance with the methods of the invention using the hTERT expression vectors of the invention should exhibit delayed senescence, as compared to untreated cells, and so be useful in transplantation therapies to treat or prevent age-related macular degeneration.

EXAMPLE 14

PROMOTER REPORTER CONSTRUCT

This example describes the construction of plasmids in which reporter genes are operably linked to hTERT upstream sequences containing promoter elements.

The vectors have numerous uses, including identification of *cis* and *trans* transcriptional regulatory factors *in vivo* and for screening of agents capable of modulating (e.g., activating or inhibiting) hTERT expression (e.g., drug screening).

Although a number of reporters may be used (e.g., firefly luciferase, β -glucuronidase, β -galactosidase, chloramphenicol acetyl transferase, and GFP and the like), the human secreted alkaline phosphatase (SEAP; Clontech) was used for initial experiments.

The SEAP reporter gene encodes a truncated form of the placental enzyme which lacks the membrane anchoring domain, thereby allowing the protein to be secreted efficiently from transfected cells. Levels of SEAP activity detected in the culture medium have been shown to be directly proportional to changes in intracellular concentrations of SEAP mRNA and protein (Berger et al., 1988, *Gene* 66:1; Cullen et al., 1992, *Meth. Enzymol.* 216:362).

Four constructs (pGRN148, pGRN150, "pSEAP2 basic" (no promoter

sequences = negative control) and "pSEAP2 control" (contains the SV40 early promoter and enhancer) were transfected in triplicate into mortal and immortal cells.

Plasmid pGRN148 was constructed as illustrated in Figure 9. Briefly, a Bgl2-Eco47III fragment from pGRN144 was digested and cloned into the BglII-NruI site of pSeap2Basic (Clontech, San Diego, CA). A second reporter-promoter, plasmid pGRN150, includes sequences from the hTERT intron described in Example 3, to employ regulatory sequences that may be present in the intron. The initiating Met is mutated to Leu, so that the second ATG following the promoter region will be the initiating ATG of the SEAP ORF.

The pGRN148 and pGRN150 constructs (which include the hTERT promoter) were transfected into mortal (BJ cells) and immortal (293) cells. All transfections were done in parallel with two control plasmids: one negative control plasmid (pSEAP basic) and one positive control plasmid (pSEAP control which contains the SV40 early promoter and the SV40 enhancer).

In immortal cells, pGRN148 and pGRN150 constructs appear to drive SEAP expression as efficiently as the pSEAP2 positive control (containing the SV40 early promoter and enhancer). In contrast, in mortal cells only the pSEAP2 control gave detectable activity. These results indicate that, as expected, hTERT promoter sequences are active in tumor cells but not in mortal cells.

Similar results were obtained using another normal cell line (RPE, or retinal pigmental epithelial cells). In RPE cells transfected with pGRN150 (containing 2.2 KB of upstream genomic sequence), the hTERT promoter region was inactive while the pSEAP2 control plasmid was active.

As noted *supra*, plasmids in which reporter genes are operably linked to hTERT upstream sequences containing promoter elements are extremely useful for identification and screening of telomerase activity modulatory agents, using both transient and stable transfection techniques. In one approach, for example, stable transformants of pGRN148 are made in telomerase negative and telomerase positive cells by cotransfection with a eukaryotic selectable marker (such as *neo*) according to Ausubel et al., 1997, *supra*. The resulting cell lines are used for screening of putative telomerase modulatory agents, for example, by comparing hTERT-promoter-driven

expression in the presence and absence of a test compound.

The promoter-reporter (and other) vectors of the invention are also used to identify trans- and cis-acting transcriptional and translational regulatory elements. Examples of cis-acting transcriptional regulatory elements include promoters and enhancers of the telomerase gene. The identification and isolation of cis- and trans-acting regulatory agents provide for further methods and reagents for identifying agents that modulate transcription and translation of telomerase.

EXAMPLE 15

SUBCELLULAR LOCALIZATION OF hTERT

A fusion protein having hTERT and enhanced green fluorescent protein (EGFP; Cormack et al., 1996, *Gene* 173:33) regions was constructed as described below. The EGFP moiety provides a detectable tag or signal so that the presence or location of the fusion protein can be easily determined. Because EGFP-fusion proteins localize in the correct cellular compartments, this construct may be used to determine the subcellular location of hTERT protein.

A. Construction of pGRN138

A vector for expression of an hTERT-EGFP fusion protein in mammalian cells was constructed by placing the EcoRI insert from pGRN124 (see Example 6) into the EcoRI site of pEGFP-C2 (Clontech, San Diego, CA). The amino acid sequence of the fusion protein is provided below. EGFP residues are in bold, residues encoded by the 5' untranslated region of hTERT mRNA are underlined, and the hTERT protein sequence is in normal font.

MVSKGEELFTGVVPILVELDGDVNGHKFSPVSGEGEGDATYGKLTTLKFICTTGKLPVPWPT
LVTTLTLYGVQCFSRYPDHMKQHDFPKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTL
VNRIELKGIDFKEDGNILGHKLEYNINSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLA
DHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDMVLLFVTAAGITLGMDELYKS
GRTQISSSSFEFAAASTORCVLLRTWEALAPATPAMPRAAPRCRAVRSLLRSHYREVLPLA
TFVRRLGPGQWRLVQRGDPAAFRALVAQCLVCPWDARPPPAAPSFRQVSCLELVARVL
QRLCERGAKNVLAFGFALLDGARGGPPEAFTTSVRSYLPNTVTDALRGSGAWGLLLRRVG
DDVLVHLLARCALFVLVAPSCAYQVCGPPLYQLGAATQARPPPHASGPRRRLGCERAWNH
SVREAGVPLGLPAPGARRRGGASRSRLPLPKRPRRGAAPEPERTPVGQGSWAHPGRTRGP
SDRGFCVVSPPAPAEATSLEGALSGTRHSHPSVGRQHHAGPPSTSRPPRPWDTPCPPVY
AETKHFYSSGDKEQLRPSFLLSSLRPSLTGARRLVETIFLGSRPWMPGTPRRRLPRLPQR

YWQMRPLFLELLGNHAQCYPYGVLLKTHCPLRAAVTPAAGVCAREKPOGSVAAPEEEDTDP
 RRLVQLLRQHSSPWQVYGFVRACLRRLVPPGLWGSRHNNRRFLRNTKKFISLGKHAKLSL
 QELTWKMSVRDCAWLRSPGVGCVPAAEHRLREEILAKFLHWLMSVYVVELLRSFYVTE
 TTFQKNRLFFYRPSVWSKLSIGIRQHLKRVQLRELSEAEVRQHREARPALLTSRLRFIP
 5 KPDGLRPIVNMDYVVGARTFRREKRAERLTSRVKALFSVLNYERARRPGLLGASVLGLDD
 IHRAWRTFVLRVRAQDPPPELYFVKVDVTGAYDTIPQDRLTEVIASIIKPQNTYCVRRYA
 VVQKAAHGHVRKAFKSHVSTLTDLPYMRQFVAHLQETSPLRDAVVIEQSSSLNEASSGL
 FDFVLRFMCHHAVRIRGKSYVQCQGIPOGSILSTLLCSLCYGD MENKLFAGIRRDGLLLR
 LVDDFLLVTPHLTHAKTFLRTLVRGVPEYGCVVNLRKTVVNFPEDEALGGTAFVQMPAH
 10 GLFPWCGLLLDTRTLEVQSDYSSYARTSIRASVTFRNGFKAGRNMRRKLFGLRLKCHSL
 FLDLQVNSLQTVCTNIYKILLQAYRFHACVLQLPFHQQVWKNPTFFLRVISDTASLCYS
 ILKAKNAGMSLGAKGAAGPLPSEAVQWLCHQAFLLKLTRHRVTYVPLLGSLRTAQQLSR
 KLPGTTLTALEAAANPALPSDFKTILD

- 15 Other EGFP fusion constructs can be made using partial (e.g., truncated) hTERT coding sequence and used, as described *infra*, to identify activities of particular regions of the hTERT polypeptide.

B. Nuclear Localization and Uses of pGRN138

- 20 Transfection of NIH 293 and BJ cells with pGRN138 confirmed the nuclear localization of recombinantly expressed hTERT. Cells were transfected with pGRN138 (EGFP-hTERT) and with a control construct (expressing EGFP only). Nuclear localization of the EGFP-hTERT is apparent in both cell types by fluorescence microscopy. As noted *supra*, the pGRN138 hTERT-GFP fusion protein supports
- 25 reconstitution of telomerase activity in both an *in vitro* transcription translation system and *in vivo* when transfected into BJ cells.

- The hTERT-EGFP fusion proteins (or similar detectable fusion proteins) can be used in a variety of applications. For example, the fusion construct described in this example, or a construct of EGFP and a truncated form of hTERT, can be used to
- 30 assess the ability of hTERT and variants to enter a cell nucleus and/or localize at the chromosome ends. In addition, cells stably or transiently transfected with pGRN138 are used for screening compounds to identify telomerase modulatory drugs or compounds. Agents that interfere with nuclear localization or telomere localization can be identified as telomerase inhibitors. Tumor cell lines stably expressing EGFP-hTERT
- 35 can be useful for this purpose. Potential modulators of telomerase will be administered to these transfected cells and the localization of the EGFP-hTERT will be assessed. In

addition, FACS or other fluorescence-based methods can be used to select cells expressing hTERT to provide homogeneous populations for drug screening, particularly when transient transfection of cells is employed.

In other applications, regions of the hTERT can be mutagenized to
5 identify regions (e.g., residues 193-196 (PRRR) and 235-240 (PKRPRR)) required for nuclear localization, which are targets for anti-telomerase drugs (telomerase activity modulators). Other applications include:

use of the fusion protein as a fluorescent marker of efficient cell
transfection for both transient transfection experiments and when establishing stable
10 cell lines expressing EGFP-hTERT;

expression of an hTERT-EGFP fusion with mutated nuclear localization signals (deficient for nuclear localization) in immortal cells so that the hTERT mutant-EGFP scavenges all the hTR of the immortal cells, retaining it in the cytoplasm and preventing telomere maintenance; and

15 use as a tagged protein for immunoprecipitation.

EXAMPLE 16

EFFECT OF MUTATION ON TELOMERASE CATALYTIC ACTIVITY

This example describes hTERT variant proteins having altered amino
20 acids and altered telomerase catalytic activity. Amino acid substitutions followed by functional analysis is a standard means of assessing the importance and function of a polypeptide sequence. This example demonstrates that changes in the reverse transcriptase (RT) and telomerase (T) motifs affect telomerase catalytic activity.

Conventional nomenclature is used to describe mutants: the target
25 residue in the native molecule (hTERT) is identified by one-letter code and position, and the corresponding residue in the mutant protein is indicated by one-letter code. Thus, for example, "K626A" specifies a mutant in which the lysine at position 626 (i.e., in motif 1) of hTERT is changed to an alanine.

30 A. Mutation of hTERT FFYxTE Motif

In initial experiments, a vector encoding an hTERT mutant protein.

"F560A," was produced in which amino acid 560 of hTRT was changed from phenylalanine (F) to alanine (A) by site directed mutagenesis of pGRN121 using standard techniques. This mutation disrupts the TRT FFYxTE motif. The resulting F560A mutant polynucleotide was shown to direct synthesis of a full length hTRT protein as assessed using a cell-free reticulocyte lysate transcription/translation system in the presence of ³⁵S-methionine.

When the mutant polypeptide was co-translated with hTR, as described in Example 7, no telomerase activity was detected as observed by TRAP using 20 cycles of PCR, while a control hTRT/hTR cotranslation did reconstitute activity. With 30 cycles of PCR in the TRAP assay, telomerase activity was observable with the mutant hTRT, but was considerably lower than the control (wild-type) hTRT.

B. Additional Site-Directed Mutagenesis of hTRT Amino Acid Residues

Conserved amino acids in six RT motifs were changed to alanine using standard site directed mutagenesis techniques (see, e.g., Ausubel, *supra*) to assess their contribution to catalytic activity. The mutants were assayed using IVR telomerase using the two step conventional TRAP assay detailed in example 7.

The K626A (motif 1), R631A (motif 2), D712A (motif A), Y717A (motif A), D868A (motif C) mutants had greatly reduced or undetectable telomerase activity, while the Q833A (motif B) and G932A (motif E) mutants exhibited intermediate levels of activity. Two mutations outside the RT motifs, R688A and D897A, had activity equivalent to wild type hTRT. These results were consistent with analogous mutations in reverse transcriptases (Joyce et al., 1994, *Ann. Rev. Biochem.* 63:777) and are similar to results obtained with Est2p (see Lingner, 1997, *Science* 276:561). The experiments identify residues in the RT motifs critical and not critical for enzymatic activity and demonstrate that hTRT is the catalytic protein of human telomerase. The mutations provide variant hTRT polypeptides that have utility, e.g., as dominant/negative regulators of telomerase activity.

Amino acid alignment of the known TRTs identified a telomerase-specific motif, motif T (see *supra*). To determine the catalytic role of this motif in hTRT, a six amino acid deletion in this motif (Δ 560-565: FFYxTE), was

constructed using standard site directed mutagenesis techniques (Ausubel, *supra*). The deletion was assayed using IVR telomerase using the two step conventional/TRAP assay detailed in Example 7. The Δ 560-565 mutant had no observable telomerase activity after 25 cycles of PCR whereas wild type hTERT IVR telomerase produced a strong signal. Each amino acid in each residue in motif T was examined independently in a similar manner; mutants F560A, Y562A, T564, and E565A retained intermediate levels of telomerase activity, while a control mutant, F487A, had minimal affect on activity. Notably, mutant F561A had greatly reduced or undetectable telomerase activity, while activity was fully restored in its "revertant", F561A561F. F561A561F changes the mutated position back to its original phenylalanine. This is a control that demonstrates that no other amino acid changes occurred to the plasmid that could account for the decreased activity observed. Thus, the T motif is the first non-RT motif shown to be absolutely required for telomerase activity.

Motif T can be used for identification of TRTs from other organisms and hTERT proteins comprising variants of this motif can be used as a dominant/negative regulator of telomerase activity. Unlike most other RTs, telomerase stably associates with and processively copies a small portion of a single RNA (i.e. hTR), thus motif T can be involved in mediating hTR binding, the processivity of the reaction, or other functions unique to the telomerase RT.

Example 17

SCREENING FOR TELOMERASE ACTIVITY MODULATORS USING RECOMBINANTLY EXPRESSED TELOMERASE COMPONENTS

This example describes the use of *in vitro* reconstituted telomerase for screening and identifying telomerase activity modulators. The assay described is easily adapted to high-through-put methods (e.g., using multiple well plates and/or robotic systems). Numerous variations on the steps of the assay will be apparent to one of skill in the art after review of this disclosure.

Recombinant clones for telomerase components (e.g., hTERT and hTR) are transcribed and translated (hTERT only) in an *in vitro* reaction as follows and as described in Example 7 *supra*, using the TNT® T7 Coupled Reticulocyte lysate system

(Promega), which is described in U.S. Patent No. 5,324,637, following the manufacturer's instructions:

| | <u>Reagent</u> | <u>Amount per reaction (μL)</u> |
|----|-----------------------------------|---------------------------------|
| 5 | TNT Rabbit Reticulocyte lysate | 25 |
| | TNT reaction buffer | 2 |
| | TNT T7 RNA Pol. | 1 |
| | AA mixture (complete) | 1 |
| | Prime RNase inhibitor | 1 |
| 10 | Nuclease-free water | 16 |
| | XbaI cut pGRN121 [hTRT] (0.5 μg) | 2 |
| | FspI cut pGRN164 [hTR] (0.5 μg) | 2 |

- 15 The reaction is incubated at 30°C for 2 hours. The product is then purified on an ultrafree-MC DEAE filter (Millipore).

The recombinant telomerase product (IVRP) is assayed in the presence and absence of multiple concentrations of test compounds which are solubilized in DMSO (e.g. 10 μM - 100 μM). Test compounds are preincubated in a total volume of 25 μL for 30 minutes at room temperature in the presence of 2.5 μL IVRP, 2.5% DMSO, and 1X TRAP Buffer (20 mM Tris-HCl, pH 8.3, 1.5mM MgCl₂, 63 mM KCl, 0.05%Tween20, 1.0 mM EGTA, 0.1 mg/ml Bovine serum albumin). Following the preincubation, 25 μL of the TRAP assay reaction mixture is added to each sample. The TRAP assay reaction mixture is composed of 1X TRAP buffer, 50μL dNTP, 2.0 μg/ml primer ACX, 4 μg/ml primer U2, 0.8 attomol/ml TSU2, 2 units/50μl Taq polymerase (Perkin Elmer), and 2 μg/ml [³²P]5'-end-labeled primer TS (3000Ci/mmol). The reaction tubes are then placed in the PCR thermocycler (MJ Research) and PCR is performed as follows: 60 min at 30°C, 20 cycles of {30 sec at 94°C, 30 sec. at 60°C, 30 sec. at 72°C}, 1 min at 72°C, cool down to 10°C. The TRAP assay is described, as noted *supra*, in U.S. Patent No. 5,629,154. The primers and substrate used have the sequences: TS Primer (5'-AATCCGTCGAGCAGAGTT-3'): ACX Primer

(5'-GCGCGG[CTTACC]3CTAACC-3'); U2 primer

(5'-ATCGCTTCTCGGCCTTTT-3'); TSU2

(5'-AATCCGTCGAGCAGAGTTAAAAGCCGAGAAGCGAT-3')

After completion of the PCR step, 4 µl of 10X loading buffer containing
5 bromophenol blue is added to each reaction tube and products (20 µl) are run on a
12.5% non-denaturing PAGE in 0.5X TBE at 400 V. The completed gel is
subsequently dried and the TRAP products are visualized by Phosphorimager or by
autoradiography. The telomerase activity in the presence of the test compound is
measured by comparing the incorporation of label in reaction product to a parallel
10 reaction lacking the agent.

The following clones described in the Examples have been deposited
with the American Type Culture Collection, Rockville, MD 20852, USA:

15 Lambda phage λ 25-1.1 ATCC accession number 209024
 pGRN121 ATCC accession number 209016
 Lambda phage λGΦ5 ATCC accession number 98505

20 The present invention provides novel methods and materials relating to
hTERT and diagnosis and treatment of telomerase-related diseases. While specific
examples have been provided, the above description is illustrative and not restrictive.
Many variations of the invention will become apparent to those of skill in the art upon
review of this specification. The scope of the invention should, therefore, be
25 determined not with reference to the above description, but instead should be
determined with reference to the appended claims along with their full scope of
equivalents.

All publications and patent documents cited in this application are
incorporated by reference in their entirety for all purposes to the same extent as if each
30 individual publication or patent document were so individually denoted.

CLAIMS:

1. An isolated, substantially pure, or recombinant protein preparation of a human telomerase reverse transcriptase (hTERT) protein, or a variant thereof, or a fragment thereof.
2. An isolated, substantially pure, or recombinant hTERT protein, said protein characterized by having an amino acid sequence with at least 75% sequence identity to the hTERT protein of Figure 17, or a variant thereof, or a fragment thereof.
3. A protein of claim 1 that has the sequence set forth in Figure 17.
4. An isolated, synthetic, substantially pure, or recombinant polynucleotide comprising a nucleic acid sequence that encodes an hTERT protein or fragment thereof.
5. A polynucleotide of claim 4 that has a sequence as set forth in Figure 16.
6. The use of a polynucleotide that is at least ten nucleotides to 10 kb in length and comprises a contiguous sequence of at least ten nucleotides that is identical or exactly complementary to a contiguous sequence in a naturally occurring hTERT gene or hTERT mRNA in assaying or screening for an hTERT gene sequence or hTERT mRNA.
7. The use of a polynucleotide that is at least ten nucleotides to 10 kb in length and comprises a contiguous sequence of at least ten nucleotides that is identical or exactly complementary to a contiguous sequence in a naturally occurring hTERT gene or hTERT mRNA in preparing a recombinant host cell.
8. A cell comprising a polynucleotide as defined in claim 7.

9. An antibody, or binding fragment thereof, wherein the antibody or fragment specifically binds to hTERT protein.
10. A method of determining whether a compound or treatment is a modulator of an hTERT activity or expression comprising detecting a change in activity or expression in a cell, animal or composition comprising an hTERT recombinant protein or polynucleotide following administration of the compound or treatment.
11. A method of determining whether a test compound is a modulator of an hTERT activity, said method comprising the steps of:
- (a) contacting an hTERT protein of claim 1 with the test compound; and
 - (b) measuring the activity of the hTERT protein, wherein a change in the hTERT activity measured in the presence of the test compound compared to the activity in the absence of the test compound provides a determination that the test compound modulates the telomerase reverse transcriptase activity.
12. A method of preparing recombinant telomerase, said method comprising contacting a recombinant hTERT protein of claim 1 with a telomerase RNA component under conditions such that said recombinant protein and said telomerase RNA component associate to form a telomerase enzyme capable of catalyzing the addition of nucleotides to a telomerase substrate.
13. A method of detecting an hTERT gene product in a sample comprising:
- (a) contacting the sample with a probe that specifically binds the gene product, wherein the probe and the gene product form a complex, and detecting the complex; or
 - (b) specifically amplifying the gene product in the biological sample, wherein said gene product is a nucleic acid, and detecting the amplification product;
- wherein the presence of the complex or amplification product is correlated with the presence of the hTERT gene product in the biological sample.

14. A method of detecting the presence of at least one telomerase positive human cell in a biological sample comprising human cells, said method comprising the steps:

- (a) measuring the amount of an hTERT gene product in said sample,
- (b) comparing the amount measured with a control correlating to a sample lacking telomerase positive cells,

wherein the presence of a higher level of the hTERT gene product in said sample as compared to said control is correlated with the presence of telomerase positive cells in the biological sample.

15. A method for diagnosing a telomerase-related condition in a patient, comprising:

- (a) obtaining a cell or tissue sample from the patient;
- (b) determining the amount of an hTERT gene product in the cell or tissue; and
- (c) comparing the amount of hTERT gene product in the cell or tissue with the amount in a healthy cell or tissue of the same type;

wherein a different amount of hTERT gene product in the sample from the patient and the healthy cell or tissue is diagnostic of a telomerase-related condition.

16. A method for increasing the proliferative capacity of a vertebrate cell in vitro by increasing expression of hTERT in the cell.

17. The use of an agent increasing expression of hTERT in the manufacture of a medicament for the treatment of a condition addressed by increasing proliferative capacity of a vertebrate cell.

18. The use defined in claim 17 wherein the medicament is for inhibiting an effect of ageing.

19. A pharmaceutical composition comprising an acceptable carrier and an hTERT protein, variant or fragment of claim 1, an hTERT antibody or binding fragment of claim 8, a polynucleotide encoding an hTERT protein, variant or fragment as defined in claim 1, or a nucleic acid that encodes an hTERT protein or subsequence thereof.

20. The use of an inhibitor of telomerase activity in the manufacture of a medicament for the treatment of a condition associated with an elevated level of telomerase activity within a human cell.

21. A protein, variant or fragment of any one of claims 1 to 3 for use as a pharmaceutical.

22. The use of a protein, variant or fragment of any one of claims 1 to 3 in the manufacture of a medicament.

23. The use of a protein, variant or fragment of any one of claims 1 to 3 in the manufacture of a medicament for inhibiting an effect of ageing or cancer.

24. A polynucleotide or fragment of claim 4 or claim 5 for use as a pharmaceutical.

25. The use of a polynucleotide or fragment of claim 4 or claim 5 in the manufacture of a medicament.

26. The use of a polynucleotide or fragment of claim 4 or claim 5 in the manufacture of a medicament for inhibiting an effect of ageing or cancer

27. A polynucleotide selected from:

(a) the DNA having a sequence as set forth in Figure 16;

(b) a polynucleotide of at least 10 nucleotides which hybridizes to the foregoing DNA and which codes for an hTERT protein or variant;

(c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b) and which code for an hTERT polypeptide or variant.

28. An hTERT protein, variant or fragment, substantially as hereinbefore described.
29. An hTERT protein-encoding or hTERT protein fragment-encoding nucleic acid sequence substantially as hereinbefore described.
30. An antibody or fragment thereof specifically binding to hTERT protein and substantially as hereinbefore described.
31. A method as defined in any one of claims 10 to 16 and substantially as hereinbefore described.
32. The use of any one of claims 6, 7, 17, 18, 20, 22, 23, 25, or 26 and substantially as hereinbefore described.
33. A pharmaceutical composition of claim 19 and substantially as hereinbefore described.



Application No: GB 9720890.4 Examiner: Colin Sherrington
Claims searched: 1 to 19, 21 to 31, 33 and Date of search: 15 December 1997
claim 32 (when appended to
claims 6, 7, 17, 18, 22, 23,
25 or 26)

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK CI (Ed.O): C3H(HB7E,HB7M)

Int CI (Ed.6): C12N 9/12

Other: ONLINE: WPI,CLAIMS,DIALOG/BIOTECH

Documents considered to be relevant:

| Category | Identity of document and relevant passage | Relevant to claims |
|----------|--|--------------------|
| A | WO 96/01835 A1 (GERON CORPORATION) -whole document | 1 (at least) |
| P,X | Science 1997,276,561-567 -Joachim Lingner <i>et al.</i> "Reverse Transcriptase Motifs in the Catalytic Subunit of Telomerase" | 1 (at least) |
| P,X | Science 1997,277,955-959 -Toru M.Nakamura <i>et al.</i> "Telomerase Catalytic Subunit Homologs from Fission Yeast and Human" | 1 (at least) |
| P,A | Trends in Cell Biology 1997,7,299-302 -Arthur J.Lustig "The identification of telomerase subunits: catalysing telomere research" | 1 (at least) |
| P,A | Cell 1997,90,785-795 -Matthew Meyerson <i>et al.</i> " <i>hEST2</i> , the Putative Human Telomerase Catalytic Subunit Gene, Is Up-Regulated in Tumor Cells and During Immortalization" | 1 (at least) |
| A | Current Opinion in Cell Biology -Kathleen Collins "Structure and function of telomerase" | 1 |
| A | J.Biological Chemistry 1995,270(42),24623-24626 -Maxine F.Singer " <i>Minireview</i> - Unusual Reverse Transcriptases" | 1 |

X Document indicating lack of novelty or inventive step
Y Document indicating lack of inventive step if combined with one or more other documents of same category.

& Member of the same patent family

A Document indicating technological background and/or state of the art.
P Document published on or after the declared priority date but before the filing date of this invention.

E Patent document published on or after, but with priority date earlier than, the filing date of this application.



The
Patent
Office

282

Application No: GB 9720890.4 Examiner: Colin Sherrington
Claims searched: 1 to 19, 21 to 31, 33 and Date of search: 15 December 1997
claim 32 (when appended to
claims 6, 7, 17, 18, 22, 23,
25 or 26)

| Category | Identity of document and relevant passage | Relevant to claims |
|----------|--|--------------------|
| A | Science 1995,269,1236-1241 -Junli Feng <i>et al.</i> "The RNA Component of Human Telomerase" | 1 |

| | | | |
|---|---|---|--|
| X | Document indicating lack of novelty or inventive step | A | Document indicating technological background and/or state of the art. |
| Y | Document indicating lack of inventive step if combined with one or more other documents of same category. | P | Document published on or after the declared priority date but before the filing date of this invention. |
| & | Member of the same patent family | E | Patent document published on or after, but with priority date earlier than, the filing date of this application. |